Physiological and performance effects of a probiotic supplementation on runners under heat stress

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Abstract

Background: Interest in the role of probiotics in gut health and wellbeing has grown rapidly over the last 10 years, however research into the use of probiotics in exercise as a potential ergogenic aid is limited. Previous literature has identified several mechanisms that may be behind the beneficial effects conferred by a range of probiotic strains on host health, such as immune function, gut regulation, inflammatory responses and cognition. It appears that a few strains have shown to exert ergogenic potential although Fonterra's proprietary strains have yet to be investigated in this space. Research in this area may lead to a potential new therapeutic option for athletes in the ever expanding, multimillion dollar sport nutrition supplement industry.

Aim: To investigate if 4 weeks of *Lactocaseibacillus rhamnosus* HN001 and *Bififidobacterium animalis* ssp. *lactis* HN019 probiotic supplementation would influence exercise performance of male runners under heat stress. Further, physiological parameters, including fluid loss, core temperature and HR were also investigated during the exercise test.

Methods: In a randomised, double-blinded, crossover study, seven male runners completed 4 weeks of dual strain probiotic or placebo supplementation followed by a 1 hour running trial in a heat chamber (30°C, 50% RH) at the end of each supplementation period. Participants ran on a treadmill at 70% of their ventilatory threshold (VT) for a 45-min pre-load phase followed immediately by a 15-min self-paced time trial. HR, core temperature and fluid loss were recorded throughout the experimental trial. Participants completed a 3-week washout period in between the two supplementation periods.

Results: Four weeks of dual probiotic supplementation did not affect exercise performance in male runners under heat stress as determined by a 15-min time trial ($p=0.63$). Physiological measures were also unaffected following the supplementation period. Reliability data showed good reliability for performance (<5% CV).

Conclusion: No ergogenic effect was conferred by the combination of these two strains, *B. lactis* HN019 and *L. rhamnosus* HN001, following 4 weeks' supplementation. Physiological measures of HR, fluid loss and core temperature, taken during the experimental trial were also negligible. It is likely either the dose, small sample size, probiotic strains or combination of all three played a role in the absence of improvement to performance and physiological measures. The reliability of test-retest (CV< 5%) reflected the robust nature of the study design despite several limitations, some of which were to be expected with running a study through COVID restrictions. However, future research should explore a dose dependent relationship, alongside increasing the sample size and investigating the possible interaction between multi strain probiotic interventions.

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Abbreviations

AA	Amino acid
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
BCAA	Branched chain amino acid
bpm	Beats per minute
BUN	Blood urea nitrogen
BW	Body weight
CFU	Colony forming unit
CHO	Carbohydrate
CNS	Central nervous system
CON	Control
COVID-19	Coronavirus-19
CRP	C-reactive protein
CV	Coefficient of variation
FAO	Food and Agricultural Organisation of United Nations
FRDC	Fonterra Research and Development Centre
GI	Gastrointestinal
GIS	Gastrointestinal Syndrome
GIT	Gastrointestinal tract
GLUT-4	Glucose transporter type 4
GSRS	Gastrointestinal symptom rating scale
H^+	Hydrogen
HDL	High-density lipoproteins
HED	Human equivalent dose
HR	Heart rate
HRmax	Maximum heart rate
I-FABP	Intestinal fatty acid binding protein
IL	Interleukin
ISAPP	International Scientific Association of Probiotics and Prebiotics
ISSN	International Society of Sports Nutrition
kg	Kilogram
kJ	Kilojoule
km	Kilometre
L/min	Litres per minute
LAB	Lactic acid bacteria
Max	Maximum
METs	Metabolic equivalent
mL	Millilitre
mL/kg/min	Millilitres per kg body mass per minute
NSAIDS	Non-steroidal anti-inflammatory drugs
pDC	Plasmacytoid dendritic cells
PLA	Placebo

PRO	Probiotic
RER	Respiratory exchange ratio
RH	Relative humidity
RPE	Rating of perceived exertion
rpm	Revolutions per minute
RTI	Respiratory tract infection
SCFA	Short chain fatty acid
SD	Standard deviation
T_c	Core temperature
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TT	Time trial
URTI	Upper respiratory tract infection
USG	Urine specific gravity
VE	Ventilation
VO_2	Aerobic capacity
VO_2max	Maximum aerobic capacity
VT	Ventilatory threshold
W	Watts

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Chapter One: Introduction

Context

Through my three-year relationship with Fonterra Research and Development Centre (FRDC) as an undergraduate nutrition scholar and intern, the opportunity to be involved in this study arose. This thesis was conducted as part of a larger, FRDC-funded study that was investigating additional complex measures which will not be included here. It is unknown if the active supplementation has influenced these other parameters, such as inflammatory markers, immune regulation, and microbiome composition, as the rest of these analyses will not be completed until after the wider study is completed. Throughout this thesis, COVID-19 has impacted several components, adding an extra challenge to this thesis year. The first lockdown occurred during pilot testing of the protocol, with recruitment planned to begin in the following month. However, it was several months later when researchers were able to confirm the protocol and recruit the first participants. The second wave of COVID-19 and subsequent rise back to Alert Level 2 in October resulted in lab restrictions which meant scheduled experimental testing was unable to be carried out. Therefore, the supplementation period of several participants was extended until they could complete testing under Level 1 conditions, which in turn extended the overall study timeline.

Background

The human gut is home to ten trillion live microorganisms which make up the microbiome (Thursby & Juge, 2017). While every individual has a unique composition of these microorganisms, it is accepted that approximately 160 bacterial strains reside in the gut and belong to five phyla, of which, Bacteroidetes and Firmicutes are the most abundant (Foster & Neufeld, 2013; Qin et al., 2010; Rajilić-Stojanović & de Vos, 2014). These commensal bacteria exert effects on the gastrointestinal tract (GIT), immune system and other body functions via host-microbiota metabolic pathways to influence host health (Nicholson et al., 2012). The proposed bi-directional communication of the gut-brain axis occurs via the vagus nerve and can involve central nervous system (CNS) signalling networks, and neural pathways between the enteric system, immune system, autonomic nervous system and neuroendocrine system (Foster & Neufeld, 2013; Wallace & Milev, 2017).

The complex adult microbiome, while relatively stable, can be influenced by several factors including diet and dietary patterns, antibiotics, probiotics, and exercise ((Wosinska, Cotter, O'Sullivan, & Guinane, 2019). Diversity of the microbiome through these factors can have a significant impact on athlete health. For example, exercise has been shown to have a direct effect on the composition and diversity of the microbiome (Clarke et al., 2014; Jang et al., 2019; Monda et al., 2017), resulting in lower risk for infections, such as upper respiratory tract infections (URTI), improved immune functioning and inflammatory response. It is well accepted that dietary fibre can act as a prebiotic by being a food source for the microbiome, resulting in improved gut health. Dietary protein intake has also been shown to influence the composition of the microbiome (Sheflin, Melby, Carbonero, & Weir, 2017). Conversely, antibiotics can have a negative impact on certain bacterial species, potentially leading to a dysbiosis which can subsequently be detrimental to host health. However, probiotics are a relatively novel nutritional supplement that has been reported to exert beneficial effects on the microbiome and host health including positive immune and inflammatory effects.

Probiotics are defined by the Food and Agricultural Organisation of United Nations (FAO) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The list of bacterial strains that are used as probiotics are diverse, although the majority are the lactic acid producing bacteria (LAB), including *Lactobacillus* and *Bifidobacterium* strains. There are hundreds of strains of probiotics which all act differently on host health, including metabolic and physiological effects. It should be noted that the term "paraprobiotics/immunbiotics" refers to heat or otherwise inactivated probiotics. While no longer live, some of these strains have the ability to confer health benefits to the host, thus still act as probiotic although do not fit under the conventional FAO definition (Jäger et al., 2019).

Due to strain specificity, there are major gaps in literature, especially with lesser known probiotic strains and their possible impact on different areas of host health. *Lacticaseibacillus rhamnosus* HN001 (formerly known as *Lactobacillus rhamnosus* HN001) and *Bifidobacterium anamalis* ssp. *lactis* HN019 are two of Fonterra's proprietary probiotic strains, originally developed in the 1990's. These two strains have been well published in the immune and inflammatory space, as well as used in maternal and infant formula for benefits to skin and immunity, however, have not been investigated within the sport and exercise space.

Within a sporting context, probiotic supplementation is not a new concept as they have been used for years to help treat common athlete ailments such as upset stomach, diarrhoea and URTI (Pyne, West, Cox, & Cripps, 2015; Wosinska et al., 2019). Trained endurance athletes, especially long distance runners, are more at risk of respiratory tract infections, possibly due to an association with increased allergy risk, and gut disturbances (Robson-Ansley et al., 2012; Simons & Shaskan, 2005). The previously founded immunosuppression theory, in which athletes experience suppressed immune response due to the effects of exercise has been challenged recently, however, authors agree that prolonged, high intensity exercise may still temporarily impact immune responses negatively, especially when compared to non-athlete populations (Moreira, Delgado, Moreira, & Haahtela, 2009). Treating these common gut and respiratory illnesses with probiotics has allowed athletes to minimise sick days and continue training schedules which in turn ensures optimal performance (Cox, Pyne, Saunders, & Fricker, 2010; Kekkonen et al., 2007). However, the use of probiotic supplementation as an ergogenic aid, exerting a direct performance enhancing effect on athletes, is a novel concept that is starting to be explored more.

The sports nutrition supplement industry is a multibillion-dollar industry, with an estimated value of US$44.8 billion in 2021, with 5 year forecasts predicting an increase to over US$60 billion (PR Newswire, 2021) . Athletes are continuously looking for a physical or mental edge over competitors, therefore supplements are commonly chosen to help achieve this edge. An international survey of elite track and field athletes reported 66% used one or more nutritional supplement (Tscholl, Alonso, Dollé, Junge, & Dvorak, 2010). According to the limited data available, use of dietary supplements is also relatively common in recreational athletes (Goston & Correia, 2010; Tsitsimpikou et al., 2011). The term ergogenic aid is used to describe supplements with performance enhancing benefits. While relatively novel, the use of probiotic supplementation as an ergogenic aid is a rapidly growing sector of the sport supplement industry (Coqueiro, de Oliveira Garcia, Rogero, & Tirapegui, 2017; Leite, Resende Master Student, West, & Lancha, 2019). The efficacy of probiotic supplementation remains unclear, simply due to the large number of probiotic strains being investigated and the strain specific effects. Therefore, currently no recommendations of dose, strain, or protocol for probiotic supplementation within the sports industry have been provided (Jäger et al., 2019).

At present, there have been 13 other studies that have investigated the ergogenic properties of probiotics on running, with two of these studies also conducted in warm environmental conditions (Cox et al., 2010; S. K. Gill, Teixeira, Rosado, Cox, & Costa, 2016; Huang et al., 2018; Huang, Lee, et al., 2019; Huang, Pan, Wei, & Huang, 2020; Huang, Wei, Huang, Chen, & Huang, 2019; Kekkonen et al., 2007; Lin et al., 2020; O'Brien et al., 2015; Pugh, Sparks, et al., 2019; Salehzadeh, 2015; Sawada et al., 2019; Shing et al., 2014). Results from these studies were mixed with some reporting performance or physiological adaptations, although most observed no significant improvement to exercise performance. Due to the vast nature of species, researchers have not yet explored the potential effects of *Lacticaseibacillus rhamnosus* HN001 or *Bifidobacterium anamalis* ssp. *lactis* HN019 within this exercise performance setting. The lack of current literature on certain probiotic strains as a supplement for direct performance enhancement is the rationale behind this thesis.

Recently, scientists have reclassified the *Lactobacillus* genus, dividing this diverse genus into 25 new genera (Zheng et al., 2020). *Lactobacillus rhamnosus* was included in this nomenclature change. Subsequently, *Lactobacillus rhamnosus* was changed to *Lacticaseibacillus rhamnosus* to better reflect the traits of this genus according to International Scientific Association of Probiotics and Prebiotics (ISAPP). This results in a name change for one of the Fonterra probiotic strains used in this thesis. *Lactobacillus rhamnosus* HN001 will be referred to using its new naming convention *Lacticaseibacillus rhamnosus* HN001. The abbreviation *L. rhamnosus* does not change. Furthermore, all historical literature included in this thesis will thereby refer to *Lactobacillus rhanmosus* as *Lacticaseibacillus rhamnosus* (*L. rhamnosus*).

This study aimed to investigate the effects of a dual strain probiotic supplement on exercise performance in male endurance recreational runners while running in the heat. The primary measure of exercise performance was distance (km) covered in the 15-minute, self-paced time trial, following a 45-minute steady state preload to obtain physiological measures. Other measures consisted of physiological parameters including core temperature, HR (heart rate), and fluid loss. **Chapter 2** provides a comprehensive literature review into the background of probiotics in exercise and continues to explore possible mechanisms behind some of the previously reported effects of certain probiotic strains on physical activity in both animals and humans. The review concludes with a look into previous literature regarding the impact of Fonterra's proprietary strains in other areas such as the gut microbiota and upper respiratory tract health in adults and children. This leads on to the aims and hypotheses in **Chapter 3**. Study design and methods are detailed in **Chapter 4** and analysis of data is documented in the results sections (**Chapter 5**). Following the results, **Chapter 6** provides the discussion. Finally, conclusions (**Chapter 7**) are drawn from the discussion, with recommendations for practical applications and future direction discussed in **Chapter 8**.

Chapter Two: Literature Review

2.1 Introduction

This review focuses on the emerging evidence of the effects of probiotic supplementation on sport and exercise performance, together with an overview of the commonly proposed mechanisms of action, importance of including heat stress in the experimental protocol, and a potential role for Fonterra's proprietary probiotic strains in this space. Until recently, the major focus of research into probiotic supplementation of active and athletic populations was GIT disorder and immune modulation. However, interest in the direct effect of probiotics on exercise performance is rapidly increasing. Although novel research on probiotics and sport performance in humans first began around 2006 (Clancy et al., 2006); 21 out of the 23 human studies reviewed here were published in the last 10 years. Similarly, animal model studies first appeared in 2008, and have been used to support evidence of ergogenic effects of certain probiotic strains in exercise performance of the human population. Animal models allow safety and efficacy aspects of trials to be established prior to human studies. The animal model studies included in this review are relevant and form a basis of rationale for beneficial effects to exercise performance conferred by probiotic supplementation.

2.2 Methods

A literature search was conducted using Google Scholar and PubMed database between November 2019 and November 2020. The searches included a combination of the following keywords: "probiotic", "athlete", "performance", "exercise', "capacity", "heat stress", "sport" and "supplement". Further studies were identified through the references of key papers. Inclusion criteria were studies with probiotic intervention (including paraprobiotic), aerobic exercise performance measures, healthy participants, including both humans and rodents, and written in English. Both human trials and animal models were included. All aerobic exercise modes were considered, however, ultra-endurance which is defined as exercise lasting more than 4 hours, was excluded (Kreider, 1991). Human populations included in the search were healthy participants, with varied training status (from untrained to athlete), involved in team sports, swimming, cycling or running modes of exercise who followed a probiotic (or paraprobiotic) supplementation intervention and completed an aerobic exercise performance test. It should be acknowledged that while resistance trained and recovery aspects of exercise have been shown to have positive outcomes for performance following probiotic supplementation, these types of studies were excluded from the search as they are out of scope for this acute, aerobic performance focussed review. See Jäger et al. (2019) or Section 2.3 of Marttinen, Ala-Jaakkola, Laitila, and Lehtinen (2020) review for further discussion on probiotic and outcomes with resistance trained populations.

2.3 Performance Outcomes

2.3.1 Animal models

Animal studies used young, untrained mice and rats, although they often familiarised the animals to the performance test with practice for 5-7 days leading up to the test (Chaves et al., 2018; Chen et al., 2016; Hsu et al., 2018; Huang, Hsu, Huang, Liu, & Lee, 2020; Lee et al., 2019; Lee et al., 2020; Soares et al., 2019). Exercise performance tests that were investigated included swimming, and treadmill running. Five studies found benefits of probiotic supplementation on mice on swim time to fatigue (Chen et al., 2016; Hsu et al., 2018; Huang, Hsu, et al., 2020; Lee et al., 2019), and one study found no benefit to rats on swim time to fatigue (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008). Chen et al. (2016) investigated six weeks of *Lactiplantibacillus plantarum* TWK10 (LP10) supplementation at 0, 2.05×10^8, or 1.03×10^9 CFU/kg/day doses in mice. They found swim time significantly increased in a dose dependent manner. They also found slow twitch Type 1 muscle

fibre numbers in the gastrocnemius muscle increased despite absence of exercise training intervention, and in addition there was dose-dependent reduction in fatigue-associated markers, creatine kinase, lactate and serum glucose levels. A more recent study by Lee et al. (2020) supported these findings, with 4 weeks of *Ligilactobacillus salivarius* ssp*. Salicinius* SA-03 supplementation resulting in significant dose dependent effect on exhaustive swim time, suggesting different strains may have similar effects on endurance capacity in animal models. The same researchers had previously investigated 28 days supplementation of *Bifidobacterium longum* ssp*. longum* OLP-01 at 0, 2.05×10^9, 4.10×10^9, and 1.03×10^{10} CFU/kg/day doses in mice (Lee et al., 2019). They similarly observed a dose dependent increase in time to exhaustion swim test. They also found a decrease in lactate and ammonia levels. Similar results were reported by Huang, Hsu, et al. (2020) who supplemented male mice with OLP-1 (1.03 x 1010 CFU/day), combined with exercise training, for 6 weeks. Notably, the OLP-1 only group and the exercise only groups independently did not produce the same significant performance improvements, which suggests a synergistic effect of the two interventions on performance outcomes in male mice. Hsu et al. (2018) investigated kefir as the probiotic delivery vehicle over 28 days at a dosage of 0, 2.15, 4.31, and 10.76 g/kg/day. The kefir contained *Lactobacillus helveticus* DSM 32787 (LH43), *Lacticaseibacillus paracasei* DSM 32785 (LPC12), *Lactobacillus rhamnosus DSM 32786* (LRH10), and *Streptococcus thermophilus* DSM 32788 (ST30). They showed dose dependent improvements in endurance swim performance (Hsu et al., 2018). Levels of serum ammonia were attenuated in the kefir groups, and muscle glycogen increased in mice in the highest kefir dose group (10.76g/kg/d). However, Desbonnet et al. (2008) did not find any performance enhancement in rats forced swim performance with two weeks of *Bifidobacteria infantis* 35624 supplementation at 1×10^{10} CFU/day.

Animal studies using rats as the model and treadmill running performance were less likely to find beneficial effect. One study found benefits of probiotic supplementation of rats on treadmill running (Soares et al., 2019), and two studies found no benefit (Chaves et al., 2018; Lollo et al., 2012). Notably, Soares et al. (2019) carried out a 10-day intervention supplementing 3×10^8 CFU/kg/day of probiotic yeast, *Saccharomyces boulardii*, by gavage in male Wistar rats. They found significant improvement in incremental speed test on a treadmill; 8 minutes longer run to fatigue time, 12.4% increase in maximum speed attained and 12.7% increase in VO_2max compared to the control group. In contrast, Chaves et al. (2018) did not find benefit in run to fatigue at 85% maximum capacity when they supplemented rats with 2 mL of fermented milk containing whey protein, 7.9×10^8 *Bifidobacterium animalis* BB12 and pomegranate five times per week for six weeks. Neither did Lollo et al. (2012) find benefit in time to fatigue in incremental treadmill test following daily supplementation with probiotic cheese containing 5×10^6 to 6×10^7 CFU/g of cheese of *Lactobacillus acidophilus* LA14 and *Bifidobacterium longum* BL05 for two weeks (Lollo et al., 2012).

Comparisons are difficult to make in the animal studies due to the differing interventions, dose and duration, exercise mode and performance test, and subject species. It is important to note that probiotic benefits are strain specific and their responses are also likely specific to the animal species being used as the biological model. Therefore, findings from animal studies are to be taken with caution, and likely not representative of similar physiological outcomes in human trials. Thus, further work is required using these specific strains in human trials in order to confirm performance effects in human populations. Interestingly, Lee et al. (2019) and (Lee et al., 2020) both used probiotic strains of human origin, isolated from the microbiome of a woman who won the gold medal at the 2008 Beijing Olympics for weightlifting. According to the authors the application of human origin strain was superior to other animal or plant-based strains. However, it appears that physiological changes in fatigue markers, endurance capacity and therefore exercise performance

have been observed across several animal studies, including different probiotic strains, of varying origins, and vehicles.

Table 1. Summary of findings from studies investigating probiotic supplementation and direct effects on sport and exercise performance in Animal Models

Reference	Subject group/design	Probiotic supplementation	Dose/intervention protocol	Dietary restrictions	Training status	Exercise test conditions	Direct performance outcomes
(Desbonnet et al., 2008)	Male adult Sprague-Dawley rats (n=20). Animal model control trial with 2 groups	*Bifidobacteria infantis* 35624	PRO: 1 x 10^{10} CFU/day, consumed orally as a dissolved powder in 100ml drinking water in the morning for 14 days. CON: vehicle only	Standard rat chow and water *ad libitum*	Untrained, no training intervention	Forced swim test, involving 3 sessions of 5 minutes in the tank (total 15 minutes) at day 3 and day 14	No significant difference in forced swim performance (swim, climbing, immobility scores) between groups at day 3 or day 14
(Lollo et al., 2012)	Male Wistar rats, aged 21 days (n=32). Animal model control trial with 4 groups	Probiotic cheese with *Lactobacillus acidophilus* LA14 and *Bifidobacterium longum* BL05	PRO: 5 x 10^6 to 6 x 10^7 CFU/g of cheese. 20g Minas fresh cheese/day for 2 weeks. CON: Conventional Minas fresh cheese with Lactobacillus lactis starter culture, 20g/day	Commercial chow and water *ad libitum*	Untrained, familiarised for 10 mins day before	Exhaustive run test on rodent treadmill with increasing speed at 90 minutes and 150 minutes until exhaustion	No direct change to performance parameters
(Chen et al., 2016)	Male Institute of Cancer Research mice aged 6 weeks (n=24). Animal model	*Lactiplantibacillus plantarum* TWK10	PRO: 2.05 x 10^8 or 1.03 x 10^9 CFU/kg, daily oral administration for six weeks, derived from	Standard chow and water *ad libitum*	No training intervention	Low force forelimb grip strength, swim to exhaustion test with constant loads carried by mice	Dose dependent effect on endurance swim time and grip strength ($p<0.001$)

	control trial with 3 groups		HED. CON: solution equivalent			at 5%BW until >7 sec resurface time	
(Hsu et al., 2018)	Male Institute of Cancer Research mice aged 6 weeks (n=32). Animal model control trial with 4 groups	*Kefir, inoculated using multistrain culture: Lactobacillus rhamnosus, Limosilactobacillus fermentum, Lactobacillus. helveticus, Lacticaseibacillus paracasei, Streptococcus thermophilus*	PRO: 2.15, 4.31, or 10.76g kefir per kg/day orally administered for 28 days. CON: glucose water with same caloric content	Standard chow and water *ad libitum*	Two-week acclimation, no training intervention	Exhaustive swim test, forelimb grip strength, lactate 10 min swim test	Statistically significant dose dependent effect of kefir supplement on endurance swim performance and grip strength
(Chaves et al., 2018)	Male Wistar rats, aged 10 weeks (n=24). Animal model control trial with 4 groups	*Bifidobacterium animalis* ssp. *lactis* BB-12	PRO: 7.9 x 10^8 CFU/day, consumed orally, 5 times/week, as 2mL fermented milk drink supplemented with whey protein and pomegranate juice for 6 weeks. CON: no milk drink supplementation	Standard rat chow and water *ad libitum*	Untrained, familiarised for 1 week using electrical stimulation	Incremental load test used to test performance via max speed, time and distance, starting at 6m/minute at 0% increasing by 3m/minute every 3 mins until exhaustion, followed by continuous aerobic test at	No significant differences in exhaustive tests between groups, and no significant difference in continuous 85% exercise capacity for fermented milk group compared to exercise only control group

						85% of ILT max speed	
(Lee et al., 2019)	Male Institute of Cancer Research mice aged 7 weeks (n=40). Animal model control trial with 4 groups	*Bifidobacterium longum* ssp. l*ongum* OLP-01	PRO: 2.05 x 10^9, 4.10 x 10^9, or 1.03 x 10^{10} CFU/kg, administered via oral gavage daily for 4 weeks, derived from HED. CON: phosphate buffered saline vehicle only	Standard chow and water *ad libitum*	Untrained, no training interventio n	Low force forelimb grip strength, swim to exhaustion test with constant loads carried by mice at 5%BW until >7 sec resurface time	Dose dependent improvements in both absolute and relative grip strength ($p<0.0001$), endurance swim time increased significantly in dose dependent manner
(Soares et al., 2019)	Male Wistar rats, aged 11 weeks (n=26). Animal model control trial with 2 groups	Saccharomyces boulardii	PRO: 3 x 10^8 CFU/kg/day, 0.1mL administered daily through oral gavage for 10 days. CON: 0.1 mL saline	Standard chow and water *ad libitum*	Untrained, no training interventio n, familiarised for 5 days using light electrical stimulation	VO_2 measured using in direct calorimetry, Time to fatigue test at 24° on purpose-built treadmill until subject received 10 second electrical stimulus, max aerobic speed and external work obtained	No changes to resting VO_2 or mechanical efficiency, but longer run to fatigue time (8 minutes), increased max speed and external work with supplementation, 12.7% increase to VO_2max at fatigue vs control, all outcomes noted as moderate

(Lee et al., 2020)	Male ICR mice (6 weeks old) divided into 4 dosage groups (n=10 per group). Intervention study with pre and post testing	*Ligilactobacillus salivarius* ssp. *salicinius* SA-03	4 dosage groups: 0 CFU/kg (Control; PBS), 2.05 x 109CFU/kg, 4.10 x 109 CFU/kg, 1.03 x 1010 CFU/kg provided by oral gavage daily for 4 weeks - converted from HED	Ad libitum water and standard chow provided during the trial	Untrained, no training interventio n	Grip strength test using tension rod; endurance swim test weighted with 5% body weight. Fasted condition for physiological testing	Significant dose dependent effect on grip strength and exhaustive swim time (indicating endurance capacity)
(Huang, Hsu, et al., 2020)	Randomised pre-post intervention study. Male ICR mice (5 week old) assigned to 4 groups: sedentary, exercise, OLP-01 and exercise + OLP-01	*Bifidobacterium longum* ssp. *longum* OLP-01	PRO group: 1.03 x 1010 CFU/kg for 6 weeks via oral gavage - converted from HED	Ad libitum water and standard chow provided during the trial	Exercise training on treadmill for 6 weeks (using shock motivation)	Low force forelimb grip strength, swim to exhaustion test with constant loads carried by mice at 5%BW	Improved endurance capacity (swim to exhaustion) and grip strength in OLP-1 + exercise group

2.3.2 Human trials

Majority of the intervention studies in humans focused on endurance athletes and endurance-based exercise performance measures including triathletes, recreational marathon runners, Division 1 swimmers and highly trained cyclists. Several studies reported improvements in performance outcomes of endurance runners and swimmers after probiotic supplementation (Huang et al., 2018; Huang, Lee, et al., 2019; Huang, Pan, et al., 2020; Huang, Wei, et al., 2019; Salarkia, Ghadamli, Zaeri, & Rad, 2013; Salehzadeh, 2015; Shing et al., 2014). Additionally, mixed results for exercise performance measures have been reported for a handful of studies that investigated probiotic supplementation in untrained participants, and team sport athletes. Further details, including supplementation period, dose, species and strain, study restrictions, exercise performance test and performance outcomes, can be found in Table 2.

Researchers have investigated probiotic supplementation in a variety of active populations, including university team sport athletes and elite national champions. However, none of these studies have demonstrated a benefit of probiotic supplementation on exercise performance (Michalickova et al., 2017; Sashihara et al., 2013), although self-reported vigour was improved. In one study, in which elite athletes across a range of sports including karate, swimming and athletics were provided with 2 x 10^{10} CFU *Lactobacillus helveticus* Lafti 10 for 14 weeks, researchers found a significant increase in self-reported sense of vigour during an aerobic capacity test (Michalickova et al., 2017). The other study failed to demonstrate any performance benefits in populations of Kanto Gakuin University football club players (Sashihara et al., 2013) following 4 weeks probiotic supplementation. The self-reported increased vigour with *Lactobacillus helveticus* Lafti 10 supplementation is a promising outcome that requires additional investigation into a potential link to performance outcome.

In three very similar studies investigating performance outcomes of competitive swimmers, only one reported benefit to swimming performance following probiotic supplementation. Researchers found significant improvement in VO_2max, as determined by the Harvard Step test, in the probiotic group, however no significant change in 400m swim time trial (Salarkia et al., 2013). This trial supplemented young female endurance swimmer's daily yoghurt (400 mL) which contained 4 x 10^{10} CFU/mL of multiple probiotic strains for 8 weeks compared to the control group who consumed 400 mL of ordinary yoghurt. Authors suggested the reduced incidence of respiratory infections and an overall improvement in health in the yoghurt group was a contributing factor in the positive change to VO_2max performance. However, Carbuhn (2017) also reported no differences in performance outcomes based on aerobic swim test between groups and did not find any differences in respiratory illness occurrence between groups as reported by the team's doctor. Likewise, another trial by the same author which investigated 6 weeks of *Bifidobacterium longum* 35624 supplementation using similar conditions, including dose, duration, training intervention, was unable to demonstrate changes to performance outcomes in swimmers (Carbuhn et al., 2018). Therefore, the effect of *Bifidobacterium infantis* 35624 and *Bifidobacterium longum* 35624 on the performance of endurance female swimmers remains unclear, although a multistrain yoghurt supplementation may indirectly produce desirable VO_2max improvements through reduced incidence of respiratory illness.

Several studies have investigated probiotic supplementation on cycling performance outcomes, with no changes to exercise performance observed (Manfred Lamprecht & Frauwallner, 2012; Muhamad & Gleeson, 2014; Pugh, Wagenmakers, et al., 2019; Pugh et al., 2020; Strasser et al., 2016; West et al., 2011). Participants in these studies were tested by time trials, maximum performance tests, and exhaustive incremental speed tests completed on a cycle ergometer. While time trial performance did not change after four weeks of 2.5 x 10^{10} CFU/day multistrain supplementation as part of double-blind crossover trial with 14 day wash out period, a significant increase in total carbohydrate oxidation alongside a reduction in fat oxidation was observed in the probiotic group (Pugh et al., 2020). No benefits were reported for VO_2max or endurance performance during exhaustive incremental cycle tests with both single and multi-strain supplementation (M. Lamprecht et al., 2012; Strasser et al., 2016; West et al., 2011). No difference in performance or HR was reported using a 2 hour cycling protocol at 60%VO_2max was found (Muhamad & Gleeson, 2014). However, reduction in GIT symptom severity at increasing training loads, cold and flu medication use (M. Lamprecht et al., 2012; Muhamad & Gleeson, 2014; Pugh, Wagenmakers, et al., 2019; Strasser et al., 2016; West et al., 2011) and self-reported URTI incidence (Strasser et al., 2016) were reported in male athletes. Furthermore, a significant increase (7.7-fold) in faecal *Lactobacillus* numbers was reported for males, while changes to female counts were inconclusive (West et al., 2011). The authors speculated that females may require greater dosage to experience the same clinical outcomes as their male counterparts, and further investigation into gender-related response to probiotics is required.

Several studies have investigated the performance outcomes of runners following a range of probiotic interventions, and performance tests carried out on treadmills and real time events with mixed results reported (Cox et al., 2010; S. K. Gill et al., 2016; Huang et al., 2018; Huang, Lee, et al., 2019; Huang, Pan, et al., 2020; Kekkonen et al., 2007; Lin et al., 2020; O'Brien et al., 2015; Pugh, Sparks, et al., 2019; Salehzadeh, 2015; Sawada et al., 2019; Shing et al., 2014). Overall, four of these studies reported improvements in running performance measures (Huang et al., 2018; Huang, Lee, et al., 2019; Huang, Pan, et al., 2020; Lin et al., 2020; Salehzadeh, 2015; Shing et al., 2014), while the rest were unsuccessful in determining benefits to running performance following probiotic supplementation (Cox et al., 2010; S. K. Gill et al., 2016; Kekkonen et al., 2007; O'Brien et al., 2015; Pugh, Sparks, et al., 2019; Sawada et al., 2019).

Interestingly, two of these double-blind, controlled studies conducted by similar research groups, found improvements to endurance running performance of healthy and not professionally trained participants following supplementation of *L. plantarum* TWK10 for 6 weeks. In 2018, Huang and colleagues reported significant increases to exhaustive run times between placebo and probiotic groups during an exhaustive treadmill run test at 85% VO_2max (Huang et al., 2018). In support of this finding, the second study found a significant improvement in endurance exercise and muscle mass in untrained participants who also did not receive exercise training as part of the intervention (Huang, Lee, et al., 2019). A significant dose dependent improvement in endurance exercise capacity (time to exhaustion) was found in both 3 x 10^{10} and 9 x 10^{10} CFU/day supplemented groups. Interestingly, while both studies observed similar overall findings for performance, the first study which used a slightly higher dosage of 1x 10^{11} compared to the latter observed a 36% increase of exhaustive run time while increases in run time in the second study with the same performance test were

approximately 17% and 31%, for 3 x 10^{10} and 9x10^{10} dose groups respectively. This could suggest that a greater dose of TWK10 over 6 weeks affects the magnitude of endurance performance improvement in untrained populations and requires further investigation to determine a saturation point for the highest dosage to exert benefit.

Under normal laboratory conditions, three studies reported significant improvements to endurance performance despite variations in study design (Huang, Pan, et al., 2020; Huang, Wei, et al., 2019; Salehzadeh, 2015). Authors reported significant improvements in exercise performance during the 2.4km Cooper run test following 10 weeks of aerobic training and supplementation of 200 mL probiotic or ordinary yoghurt drink, containing a mix of 1 x 10^5 CFU/g of *Streptococcus Thermophilus* or *Lactobacillus delbrueckii* ssp*. Bulgaricus* (Salehzadeh, 2015). VO_2max was also significantly different between supplemented and control groups. HS-C reactive protein levels, a marker for inflammation, were reduced in the probiotic yoghurt group while HDL levels were increased. This suggests possible enhancement to cardiovascular system, thereby contributing to aerobic performance benefits.

Whereas, Huang and colleagues found significant increase to endurance performance by 130% of supplemented triathletes completing the Bruce protocol however, VO_2max measures were not found to be significantly different between the probiotic and control groups (Huang, Pan, et al., 2020). This was following 4 weeks of *L. plantarum* PS128 supplementation combined with regular training. Significant beneficial alterations to microbiota composition were identified following intervention, with the PS128 group also producing greater SCFA. The same researchers have previously suggested that *L. plantarum* PS128 supplementation acts to improve inflammatory markers such as CK in triathletes and may influence physical performance following high intensity events (Huang, Wei, et al., 2019). Forty-eight hours following a triathlon, trained triathletes, completed cycle to exhaustion test at 85% VO_2max. Endurance performance, as determined by time to exhaustion, was significantly improved in the probiotic group compared to placebo following a 3-week supplementation period and training programme.

Although no significant differences were found in running distance completed during the 12 min Cooper test between the OLP-01 probiotic and placebo groups following 5-week intervention period, the OLP-01 group showed improvements in running distance at the 3rd, 6th, 9th and 12th min compared to pre-intervention results (Lin et al., 2020). Interestingly, the proportion of beneficial bacteria genus and species was greater in the OLP-01 group after 5 weeks of supplementation, while a reduction in pathogenic probiotic strains was also observed compared to the control group.

In contrast to the findings above, one of the earlier crossover studies in this area by Cox et al. (2010) reported no significant difference in VO_2max or run time trial between placebo and *L. fermentum* VRI-003 supplemented elite male distance runners. Several studies also investigated running performance during real running events but observed no improvements in running performance (Kekkonen et al., 2007; O'Brien et al., 2015; Pugh, Sparks, et al., 2019; Sawada et al., 2019). No significant difference in marathon finish times were found following a 28-day 2.5 x 10^{10} CFU/day multistrain probiotic supplementation (Pugh, Sparks, et al., 2019). Similarly, O'Brien and colleagues found no changes in performance time of a 1.5 mile run in

athletes in training for marathon following consumption of a kefir beverage after training sessions twice weekly (O'Brien et al., 2015). This study was limited as the probiotic dosage was not reported, together with infrequent consumption of the beverage (only twice weekly). The performance test was also not very relevant to the athletes, as a 2.4km run is a relatively short distance for an athlete in training for a 42km event. Other studies using long distance field-based events as the performance measure also found there was no difference in mean run time for the probiotic group compared to placebo group (Kekkonen et al., 2007; Sawada et al., 2019). While time trials and time to exhaustion tests are commonly used for performance measures, there is evidence to suggest the reliability of results from a time trial method is greater than those from the exhaustive test (Laursen, Francis, Abbiss, Newton, & Nosaka, 2007). Another limitation is that studies that investigate real time events, such as a marathon, lack baseline measures due to logistical difficulties. For example, the participants involved in the study by Pugh and colleagues did not complete a baseline marathon run before starting the supplementation period (Pugh, Sparks, et al., 2019).

Despite finding no benefit to physical performance, one study reported microbiota diversity of athletes in the probiotic group was enhanced (Sawada et al., 2019). Some authors also reported lower severity of GI symptoms which may allow athletes to maintain their running pace for longer during endurance exercise (Kekkonen et al., 2007; Pugh, Sparks, et al., 2019), for example a significant reduction in average run pace during the third and last leg of a marathon in the placebo group was observed (Pugh, Sparks, et al., 2019). Similarly, another study found a 33% reduction in the GIT symptoms for the probiotic group during the two weeks after the marathon (Kekkonen et al., 2007).

Table 2. Summary of findings from studies investigating probiotic supplementation and direct effects on sport and exercise performance in Humans

Reference	Subject group and design	Probiotic supplementation	Dose and intervention protocol	Dietary restrictions	Training status	Exercise test conditions	Direct performance outcomes
(Kekkonen et al., 2007)	Healthy, mixed gender, recreationally trained marathon participants, aged 22-69 y (n= 119). Double blind, randomised placebo controlled parallel trial	*Lactobacillus rhamnosus* GG	PRO: 4 x 10^{10} CFU/day, consumed as two 65ml milk-based fruit drinks for 3 months, optional to consume capsules if more convenient (1 x 10^{10} CFU/day taken as two capsules. PLA: identical drink without probiotic	Antibiotics (preceding 2 months prior to study), probiotic-containing food products,	Recreationally trained runners who had completed a marathon in under 3.5/3.75 hours before this event, summertime training for at least 3 months before race	Helsinki City Marathon, with no restrictions on food/fluid throughout marathon event	No significant difference between groups for marathon time
(Cox et al., 2010)	Male elite distance runners, mean age 27.3 ± 6.4 y (n=20). Double blind, placebo-controlled crossover trial	*Limosilactobacillus fermentum* VRI-003 (PCC)	PRO: 1.2 x 10^{10} CFU/day, consumed as two gelatine capsules three times per day with food (6/day total) for 28 days	Yoghurt and other yoghurt containing products	Experienced well-trained distance runners, participating in events such as 800m to marathon	Incremental exhaustive run test on treadmill at 0% gradient until 20km/h reached then 1% gradient increase/min, VO_2max	No significant differences for VO_2max or treadmill performance time

			with 28 d washout. PLA: Micro-crystalline cellulose capsules				
(West et al., 2011)	Competitive, mixed gender cyclists, mean age 35 ± 9 y (M), 36 ± 9 y (F) (n=99). Double blinded, randomised placebo-controlled trial	*Limosilactobacillus fermentum* VRI-003 (PCC)	PRO: 1 x 10^9 CFU/day, consumed as daily capsule for 11 weeks. PLA: daily capsule containing microcrystalline cellulose	Probiotic-enriched foods (including yoghurt), probiotic supplements and antibiotics (all preceding 1 prior and throughout study period)	Normal training load during winter training, VO_2max requirement: >45ml/kg/min (female) or >50ml/kg/min (male)	Incremental cycle ergometer test measures of VO_2max, peak power output	No significant difference in peak power or VO_2max measures
(Lamprecht et al., 2012)	Male endurance trained, aged 30-45 y (n=23). Double blinded randomised placebo-controlled trial	*Multistrains: 6 Bifidobacterium, Enterococcus, Lactobacillus* and *Lactococcus* strains	PRO: 1 x 10^{10} CFU/day, consumed 1 hr before meals as 2 sachets mixed with 100-125mL plain water for 14 weeks. PLA: same powder matrix of cornstarch,	Standardized breakfast 3 hours prior to exercise session	Endurance trained athletes including cyclists, triathletes, runner, with VO_2max >45mL/kg/min, no physical training 3 days	Incremental cycle ergometer test performed at 80 rpm and increasing 20W every min from 60W until exhaustion	No differences in performance time on cycle erg (VO_2max or max performance) between groups

			maltodextrin, vegetable protein, $MgSO_4$, $MnSO_4$ and KCl without probiotic		before ex session		
(Shing et al., 2014)	Male runners, mean age 27 ±2 y (n=10). Double blind, placebo-controlled crossover trial	Multistrain: *Lactobacillus rhamnosus*, Lacticaseibacillus *casei, Lactiplantibacillus plantarum, Limosilactobacillus fermentum, Bifidobacterium lactis, Bifidobacterium breve, Bifodobacterium bifidum, Streptococcus thermophiles* (strains not specified)	PRO: 4.5 x 10^9 CFU, daily capsule for 4 weeks with 28d washout period. PLA: identical skim milk powder capsule, consumed daily	Antibiotics or probiotics (preceding 6 months), ergogenic aids (preceding 1 month)	Trained runners, not acclimatised to running in the heat, no strenuous exercise performed 24 hours prior to testing	VO_2max, ventilatory threshold (VT) and peak running speed assessed first. Time-to-fatigue run test performed on treadmill at 80% vent threshold at 35°, 40% humidity	Significant increase in run-to-fatigue time with probiotic supplementation
(Salarkia et al., 2013)	Female endurance swimmers aged 11-17 y (n=46). Randomised control trial	*Probiotic yoghurt with multistrains: Lactobacillus acidophilus, B. bacterium, Lactobacillus*	PRO: 4 x 10^{10} CFU/mL, consumed daily as 400mL of yoghurt for 8 weeks. CON:	Antibiotics (2 months preceding), other probiotics	Elite, healthy, endurance-trained athletes, can swim 3.8km under 2.5 hrs	400m free swimming test, VO_2max determined by Harvard step test protocol	No significant change to 400m swim record, however significant improvement to VO_2max for

		Delbrueckii Bulgaricus, Streptococcus Salivarius Thermnophilus	ordinary yoghurt		at least 3 times/week		supplemented group
(Sashihara et al., 2013)	Well trained, healthy male university athletes, aged <30 y (n=44). Double blind, placebo control randomized clinical trial with 3 intervention groups	*Lactobacillus gasseri* OL2809 (heat-inactivated paraprobiotic)	PRO: probiotic (1×10^{10} CFU/day) alone or probiotic + 900mg a-LA, two tablets three times a day after meals for 4 weeks. PLA: identical dextrin tablet	No reported restrictions	Highly trained, Japanese university athletes, members of football club involved in high intensity training at least 5 times per week	Strenuous 1 h cycle test on bicycle ergometer, maintained intensity at approximately 70% HRmax	No significant difference to exercise performance (outcome measures: workload and calories) between groups
(Muhamad & Gleeson, 2014)	Healthy, active university students, aged 18-25 y (n=11). Pre and post intervention trial	*Multistrains: 14 Lactobacillus, Lactococcus, Bifidobacterium* and *Streptococcus* strains	PRO: 6×10^{9} CFU/day, consumed as capsule three times a day for 30 days	Probiotics (preceding 3 months and throughout) , overnight fast prior to testing session	Active students with at least 3 endurance sessions (30 mins+) per week, no further detail regarding training during intervention period	VO_2max test, followed by acute test of prolonged exercise on cycle erg at 60% VO_2max for 2 hours (fasted, water permitted)	No significant difference in rating of perceived effort or HR between pre and post trials
(O'Brien et al., 2015)	Mixed gender healthy runners, aged 18-35 y (n=67).	Kefir beverage	PRO: $1 \times 10^{9} – 1 \times 10^{10}$ CFU/day consumed as kefir fruit base beverage within 30 mins	No details provided	Training for marathon, 15-week endurance training programme	1.5 mile time trial (no further details provided)	Significant improvement to 1.5-mile time for both exercise subgroups (kefir and control

	Placebo-controlled trial with 4 groups		after twice weekly training session for 15 weeks. PLA: matched beverage with lactose-free milk product as kefir replacement				beverage supp), no diff for kefir + no exercise subgroup
(Salehzadeh, 2015)	Male, endurance athletes, mean age 21 y (n=30). Matched, randomised control trial	Yoghurt beverage with *Streptococcus Thermophilus* or *Lactobacillus delbrueckii* ssp. *Bulgaricus* or both	PRO: 1 x 10^5 CFU/g (approx. 2.07 x 10^7 CFU/day), consumed as 200mL yoghurt drink daily for 10 weeks. CON: ordinary yoghurt drink	No details provided	10-week training programme	Cooper aerobic test (12 min, 2.4km run) with the max aerobic power (VO_2max) assessed using Balke treadmill test	Significant improvement in VO_2max and records (aerobic power) between groups
(Strasser et al., 2016)	Well trained, healthy, mixed gender, mean age 26.7y (n=33). Double blind, placebo controlled randomised clinical trial	Multispecies: 6 *Lactobacillus*, *Bifidobacterium* and *Enterococcus* strains	PRO: 1 x 10^{10} CFU/day, consumed 1 hr before breakfast as 1 sachet mixed with 100-125mL plain water for 12 weeks. PLA: matrix powder containing cornstarch,	Antibiotics, probiotics, dietary supplements, alcohol (>10g and 20g/day for female and male respectively), fermented dairy	Trained athletes, maintaining own personal training programme during study period (no further detail provided)	Incremental exhaustive cycle test on ergometer, increasing from 50/70W in 25W/minute increments until exhaustion, 20min time trial (main outcome mean	Significant increase in higher training loads for probiotic group, however no differences between groups for performance measures

			maltodextrin, vegetable protein, $MgSO_4$, $MnSO_4$ and KCl	products, standardized breakfast meal 2 hr prior to testing		power output from TT)	
(Michalickova et al., 2017)	Mixed sport, highly trained elite National/world champ winner athletes, aged 18-28 y (n=39). Double blind randomised, placebo controlled parallel trial	*Lactobacillus helveticus* Lafti L10	PRO: 2 x 10^{10} CFU/day, consumed as a daily capsule after breakfast for 14 weeks. PLA: Identical capsule containing 99% maltodextrin and 1% magnesium stearate, consumed daily	Immune system-related supplementation, multivitamins/ minerals, yoghurt and other fermented milk products, probiotic and antibiotic use (preceding 1 month prior to study)	Highly trained, National/World champions in their specific sport (badminton, triathlon, cycling, athletics, judo etc), training >11hr/week (high training load)	Aerobic capacity determined by VO_2max test on treadmill, with progressive intensity until volitional exhaustion, 90% of max HR reached and plateau in VO_2 observed despite increasing workload	No significant differences in VO_2max, run time on treadmill performance, max or recovery HR
(S. K. Gill et al., 2016)	Male highly trained endurance athletes, mean	*Lacticaseibacillus casei*	PRO: 1 x 10^{11} CFU/day, consumed as daily probiotic beverage with	Probiotics (preceding 3 months and throughout	Endurance trained athletes with experience in competitive	Exertional heat stress test completed at 60% VO_2max for 2 hours on	No significant difference to HR or RPE

	age 26 ± 6 y (n=8). Blinded, randomised crossover trial		split dose (500mL AM, 500mL PM) for 7 days with 1-month washout. PLA: nutritionally matched beverage, consumed daily	study period), alcohol and caffeine (both 72 h prior to exercise session), standardized breakfast 1-hour prior	endurance events such as trail running and triathlons, non-heat acclimatized	treadmill in controlled chamber at 34±0.4° and 32±2% humidity, with *ad libitum* water throughout test	
(Carbuhn, 2017)	Female collegiate Division I swimmers (n=17). Double blind, placebo controlled, randomised clinical trial	*Bifidobacterium infantis* 35624	PRO: 1 x10^9 CFU/ day in daily capsule form, for six weeks. PLA: identical maltodextrin capsule, consumed daily	Antibiotics (1 month preceding and throughout study period), nutritional supplements excluding multivitamin, vitamin C/D and iron, ergogenic aids (1 week preceding), food rich in probiotics (e.g. Kefir)	Highly trained swimmers participating in 6-week intensified resistance and swim training programme during off season	Aerobic swim test (500m freestyle TT) conducted in the team's normal 25m training pool	No significant difference for performance test between the two groups

(Carbuhn et al., 2018)	Female collegiate, "high level" competitive swimmers (n=17). Double blind, randomised placebo-controlled trial	*Bifidobacterium longum* 35624	PRO: 1 x 10^9 CFU/day, consumed as one daily capsule for duration of six-week study. PLA: identical maltodextrin capsule, consumed daily	Antibiotics (from 1 month preceding onwards), nutritional supplements, ergogenic aids (from 1 week preceding) throughout study, foods rich in probiotics and caffeine	"High level" competitive swimmers with considerable swimming experience, participating in intense structured training regime over off season	Aerobic 500m time trial tested 3 time points during 6-week intensive training phase	No changes in exercise performance
(Huang, Lee, et al., 2019)	Healthy, mixed gender, non-athlete participants aged 20-30 y (n=54). Double blind, randomised, placebo-controlled trial	*Lactiplantibacillus plantarum* TWK10	PRO: 3 x 10^{10} or 9 x 10^{10} CFU, 3 capsules per day taken after meals for 6 weeks. PLA: matric capsule containing maltodextrin and microcrystalline cellulose	Prebiotics, probiotics, fermented foods (e.g. yoghurt), dietary/nutritional/ergogenic supplements	Healthy, no professional athletic training, advised to maintain usual physical activity, no strenuous exercise for 3 days prior to first VO_2max test	VO_2max for reference, exercise test at fixed intensity 60% VO_2max, run to exhaustion test on treadmill at 85% VO_2max increasing 1.8km/h every 2 minutes	Dose dependent increase in endurance running performance (run to fatigue)

(Sawada et al., 2019)	Male long-distance university runners aged 18-22 y (n=49). Double blind, randomised placebo-controlled clinical trial	*Lactobacillus gasseri* CP2305 (inactivated paraprobiotic)	PRO: 1 x 10^{10} bacterial cells, consumed daily as 200mL sports isotonic beverage for 12 weeks. PLA: identical beverage without probiotic, consumed daily	LAB-enriched foods	Extensive training during experimental period for key Eikden events	Eikden road relay events	No differences in physical performance at the events between groups
(Huang, Wei, et al., 2019)	Male university-aged triathletes, (S1: n=18, S2: n=16). Double blind placebo controlled clinical trial, split into two similar studies	*Lactiplantibacillus plantarum* PS128	PRO: 3 x 10^{10} CFU/day, consumed as twice daily capsules (taken after training and before sleeping) for 4 weeks (S1) or 3 weeks (S2). PLA: microcrystalline cellulose capsules	Prebiotics, probiotics, antibiotics, fermented foods (e.g. yoghurt), dietary/nutritional supplements	No strenuous exercise 24 hrs prior to testing, trained athletes involved in specific training programme for the study	30 second Wingate test used to assess aerobic capacity, endurance tested through exhaustive test on cycle ergometer at 85% VO_2max - tested before and after triathlon	Significant increase in mean power and fatigue index as part of Wingate as well as endurance indices for probiotic group
(Pugh, Sparks, et al., 2019)	Healthy, mixed gender, recreational runners, aged 22-50 y (n=24). Double blind, randomised	*Multistrain: Lactobacillus acidophilus* CUL60 and CUL21*, Bifidobacterium bifidum*	PRO: 2.5 x 10^{10} CFU/capsule, consumed daily after first meal of the day for 28 days, with an additional	Probiotic foods e.g. fermented yoghurt, standardized meal 24 hour prior	Recreationally trained runners who had completed a marathon in less than 5 hours in	Marathon, run on outdoor 400m track (105.48 laps) in 16-17°, 8-16km/hr wind	Lactate threshold, VO_2max, marathon finish time all not significant, however

	placebo-controlled trial	CUL20 and *Bifidobacterium anamalis* ssp. *lactis* CUL34	capsule taken 2 hours before race start. PLA: identical cornstarch capsule	to race as well as standardized breakfast on morning of race (supplied), race nutrition strategy provided (gels etc)	previous 2 years	speed and no rain	improved maintenance of pace in last third of race
(Pugh et al., 2020)	Trained male cyclists, mean age 23 ± 4y (n=7). Double blinded, randomised, placebo-controlled crossover trial	*Multistrain: Lactobacillus acidophilus* CUL60 and CUL21, *Bifidobacterium bifidum* CUL20, *Bifidobacterium animalis* ssp. *lactis* CUL34	PRO: 2.5 x 10^{10} CFU/day, consumed as daily capsule for four weeks with 14-day washout. PLA: visually identical capsule, no further details provided	Probiotic foods e.g. fermented foods and yoghurt, standardized meal 24 hour prior to race (no spicy food, alcohol or caffeine)	Trained cyclists	Cycle erg test at 55%Wmax for 120 minutes with constant intake of maltodextrin solution, immediately followed by time trial (100kJ)	No significant difference in time trial (100kJ) performance between groups
(Lin et al., 2020)	Well trained middle- and long-distance mixed gender runners, aged 20-30 years (n=21). Double blind, placebo-controlled trial	*Bifidobacterium longum* ssp. *longum* Olympic No. 1 (OLP-01), combined with regular structured training plan	PRO: 1.5 x 10^{10}/day, 3 capsules taken per day following each main meal for 5 weeks alongside 3-week	Nutritional supplements, Yakult, yoghurt, antibiotics, other probiotic products; alcohol	3 weeks training followed by 2 weeks of de-training	12-minute Cooper running/ walking test	No significant difference in running distance completed between groups, although running improvement was significantly improved in OLP-

			structured training plan and 2 weeks of de-training. PLA: identical capsule of maltodextrin	abstinence 1 week prior to testing			01 group at 6th, 9th and 12th minute compared to pre-intervention times
(Huang, Hsu, et al., 2020)	Double-blinded randomised intervention study with parallel groups. Male, trained triathletes at university (n=10 per each group)	*Lactiplantibacillus plantarum* PS128	PRO: 3 x10^{10} CFU/day taken as two capsules (one after training and one before sleeping) for 4 weeks PLA: Identical capsules filled with cellulose	Fermented foods, probiotics, prebiotics, smoking, alcoholic beverages	Regular training during supplementation period. No strenuous exercise in 24 hours prior to test	Exercise performance was completed on treadmill, determined by Bruce protocol	Endurance capacity significantly increased by 130% compared to placebo, although no improvement to VO2max
(Huang et al., 2018)	Double-blinded placebo-controlled clinical study Professionally untrained, health male adults, aged 20 – 40 years (n=16)	*Lactiplantibacillus plantarum* TWK10	PRO: 1 x 10^{11} CFU/day taken as one capsule after a meal PLA: Identical capsule filled with maltodextrin, deproteinised permeate whey powder, lactose and microcrystallin	Nutritional supplements for duration of study period; alcoholic drinks, probiotic products, yoghurt, or drugs during week	Avoid strenuous physical activity in three days prior to VO_2max test	VO_2max test prior to 85% VO_2max exhaustive treadmill test	Aerobic endurance capacity (determined by time to exhaustion) significantly increased compared to placebo group

			e cellulose (no probiotic)	prior to trial; consumed standardised breakfast morning of trial			

2.4 Potential mechanisms

In terms of probiotic effects on athletic performance, several mechanisms have been proposed, although evidence remains to be fully elucidated. Potential mechanisms include effects on intestinal permeability, amino acid (AA) utilisation, immunoregulatory system, short chain fatty acid substrate utilisation, mental state, fatigue-associated markers and diversification of gut microbiota. This is a complex topic; the specific benefit can vary between strains, and the strength of effect can vary between strains, dosages and supplementation period, as well, the usefulness of the health benefit to the exercise mode. Some mechanisms also rely on the transient colonisation of the live microorganism within the host gut (Monteagudo-Mera, Rastall, Gibson, Charalampopoulos, & Chatzifragkou, 2019). Gaining a greater understanding of the mechanisms by which probiotics benefit performance is crucial to providing recommendations of species and strain, dose, supplementation period and benefit to specific modes of exercise.

2.4.1 Gut microbiota diversification

Previous research has shown gut microbiome can be modified through various factors, including exercise, diet, antibiotics and probiotic use (Marchesi et al., 2016; Monda et al., 2017). For example, high intensity exercise training can cause perturbations to the intestinal ecosystem, as seen in both animal and human trials (Chaves et al., 2018; Clark & Mach, 2016). On the other hand, exercise is known to also assist advantageous modification of gut microbiota (Monda et al., 2017). A recent study highlighted the differences in microbial diversity between bodybuilders, runners and a healthy, sedentary control group, with microbiota differences presumably altered through a combination of exercise mode, diet and other factors (Jang et al., 2019). Elite rugby athletes were shown to have an improved ratio of *Bacteroidetes* to *Firmicutes* compared to the faecal profile of a non-athlete control group (Clarke et al., 2014). Additionally, the number of butyrate-producing bacteria, important to gut health, has been shown to increase with higher habitual amounts of exercise by the host, as determined by aerobic fitness (VO_2max) (Estaki et al., 2016). Therefore, the training status of participants needs to be considered as a probiotic intervention may be more likely to exert benefit in less active populations as there is more room for physiological change, specifically diversification to gut microbiome. Thus, non-trained or recreationally active people may have greater benefit from a probiotic supplement compared to highly trained people.

Microbial diversity has been suggested as an indicator of health status; however, the many factors that can influence a person's gut microbiome makes this a complex theory. Some probiotics have been found to modulate the composition of gut microbiota, such as *Bifidobacterium* supplementation, which may provide a more favourable environment for the reestablishment of lactobacillus microorganisms in the athlete's intestinal ecosystem (Chaves et al., 2018). Several studies have posited that gut microbiota diversification, increased by probiotic supplementation, could be a mechanism for benefit to athletic performance (Chaves et al., 2018; Chen et al., 2016; Hsu et al., 2018; Huang, Lee, et al., 2019; Sawada et al., 2019). It is speculated that the probiotic-induced lowered ratio of *Firmicutes* to *Bacteroidetes* can have more positive contribution to metabolic pathways which reduce fatigue risk (Hsu et al., 2018). While no authors have shown a direct link between the modification of gut microbiota and athletic performance; Sawada et al. (2019) found heat-inactivated *Lactobacillus gasseri* CP2305 increased diversification of gut microbiota and thereby alleviated fatigue and

attenuated stress in university runners. Thus, enhanced energy balance through modification of gut microbiota is an area for further consideration for exercise performance.

Regardless of the diversity, the total amount of microbiota has also been suggested to enhance endurance capacity and improve athletic performance (Chen et al., 2016). Evidence showing greater numbers of intestinal microbiota correlates with improved endurance swimming time in a murine model which supports the notion that the impact of gut microbiota on athletic performance could be as simple as ensuring an abundance of microbiota. The authors suggested that an increase in the number of microbiota may act to modulate energy utilisation of the host, with this possible underlying mechanism helping to improve liver and muscle glycogen levels, thereby increasing endurance capacity and performance (Hsu et al., 2015). Nevertheless, more research is required to explore the possible link between probiotic supplementation and athletic performance via changes to gut microbiota diversity.

2.4.2 Intestinal permeability and gut discomfort

Strenuous exercise can cause GIT disorders including flatulence, runner's diarrhoea, and bloating (Leite et al., 2019). These symptoms are more prevalent in endurance athletes involved in high intensity exercise with greater training loads. Disruption to intestinal barrier integrity is one of the ways high intensity and long duration exercise can result in GIT discomfort and impaired performance and health (Rawson, Miles, & Larson-Meyer, 2018). Redistribution of blood flow away from the GIT, towards the active muscles can cause splanchnic hypoperfusion compromising gut barrier function which may also lead to GIT symptoms (Wijck et al., 2012). The intestinal epithelia barrier works to protect the host against the external environment. This key defence mechanism relies on a structural combination of mucosa layer, antimicrobial peptides, intestinal epithelia layer, tight junction complexes, and immune cells (Vancamelbeke & Vermeire, 2017). The functioning of this barrier can determine the permeability which regulates the delivery of important nutrients and restriction of pathogens (Chelakkot, Ghim, & Ryu, 2018). Disruption to the intestinal barrier can be induced by exercise stress which increases intestinal permeability, also known as leaky gut, thereby allowing pathogens and toxins to infiltrate the host (Wijck et al., 2012). Leaky gut can increase the risk of endotoxemia and lead to an elevated immune response and GIT discomfort for the athlete (Clark & Mach, 2016). Therefore, through reduced intensity and disruption to training regimes as a result of athletes dealing with GIT symptoms, athletic performance can become impaired. Previous work has suggested probiotic supplementation influences gut permeability (Rao & Samak, 2013; Wosinska et al., 2019).

While there has been substantial interest in this area, the results on the use of probiotics as a therapeutic tool to attenuate GIT problems during exercise and benefit to performance remain mixed (Rawson et al., 2018). For example, one study found the probiotic group reported less GIT symptoms and improved maintenance of pace during the last section of a marathon (Pugh, Sparks, et al., 2019). Zonulin is used as a marker of gut permeability as this protein plays a modulatory role in tight junctions situated in between cells of the epithelial layer; increased level of zonulin in the faeces is a marker of impaired barrier integrity (Fasano, 2012). Probiotic supplementation has been demonstrated to reduce faecal zonulin levels in athletes (Lamprecht et al., 2012). In addition, an increase in tight junction proteins expressed at the intestinal barrier has been proposed as a factor in improved permeability following probiotic supplementation. An increase in the expression of tight junction proteins in young

rats following fermented milk supplementation, and in mice, probiotic supplementation facilitated rapid expression of tight junction proteins to accelerate the maturation of the immature intestinal barrier (Chaves et al., 2018; Patel et al., 2012). In seven healthy humans, an increase in two important proteins (zonula occludens and occludin) within the area of tight junction complexes was found following administration of *Lactiplantibacillus plantarum* WCSF1 supplementation (Karczewski et al., 2010). Authors suggested that this finding is important and supports the understanding that some *Lactiplantibacillus plantarum* strains act to tighten the epithelial barrier. Further, this mechanism was reported to be dependent on the homeostatic role of TLR2 signalling within the mucosal immune system, which has also been shown in an *in vitro* model (Cario, Gerken, & Podolsky, 2007; Karczewski et al., 2010). Moreover, it has been suggested that metabolites of gram-positive bacteria, such as Lactobacillus and Bifidobacterium strains, modulate the mucosal immune system through ligand binding and subsequent activation of the TLR2 pathway (Wells, Rossi, Meijerink, & van Baarlen, 2011). Therefore, this could provide the link between probiotic supplementation and enhanced intestinal barrier function.

2.4.3 Amino acid absorption and adaptation to exercise

One possible mechanism believed to underlie several performance benefits induced by probiotic supplementation is the improved gastrointestinal absorption of AA from dietary protein. Increased absorption of dietary protein may allow greater adaptations to athletes, specifically muscular mass, strength and recovery, during training periods, resulting in improved performance during subsequent exercise. Also, increased protein absorption has been postulated to help athletes including impacting satiety and causing thermogenesis, both of which can influence body composition which may be beneficial for athletic performance in some sports (Toohey et al., 2018). Multiple studies have discussed the notion of protein absorption aiding performance outcomes, however details of the exact mechanism vary between authors (Chen et al., 2016; Georges et al., 2014; Huang, Wei, et al., 2019; Jäger, Shields, et al., 2016; Toohey et al., 2018; Townsend et al., 2018).

There is evidence to suggest supplementation of probiotics can enhance AA absorption, as demonstrated by improved leucine absorption following probiotic and whey protein supplementation (Jäger, Purpura, Farmer, Cash, & Keller, 2018; Maathuis, Keller, & Farmer, 2010; Y. Wang & Gu, 2010). This is thought to be due to more efficient protease activity following probiotic ingestion (Keller, Van Dinter, Cash, Farmer, & Venema, 2017; Maathuis et al., 2010). Endurance exercise performance may benefit from enhanced branched chain amino acid (BCAA) absorption in the form of fatigue mitigation (Cheng et al., 2016). Improved efficiency of absorption may allow more rapid initiation of muscle protein synthesis to enhance muscle recovery (Buckley et al., 2010) and recovery of muscle strength following bouts of damaging exercise (Jackman et al., 2017; Jäger, Shields, et al., 2016). Evidence of this comes from *Lactiplantibacillus plantarum* PS128 supplementation which substantially increased appearance of plasma BCAA as well as enhanced exercise performance in triathletes who had completed at least 5 years of structured training (Huang, Wei, et al., 2019). Furthermore, the increase in muscle mass and number of type 1 muscle fibres in the gastrocnemius of mice, without a training intervention, indicates a possible role for probiotic supplementation to influence body composition (Chen et al., 2016). Therefore, probiotic supplementation, together with adequate protein intake, may help maximise the benefits of protein consumption for both aerobic and resistance athletes, including muscle damage recovery and hypertrophy, although more research is warranted in the future.

2.4.4 Short chain fatty acid production

Short chain fatty acids (SCFA) are metabolites produced by certain gut bacteria. SCFA, primarily consisting of acetate, propionate and butyrate, are products of bacterial fermentation of indigestible dietary fibre in the gut (Nagpal et al., 2018). The production of SCFA can be modulated by probiotic supplementation due to restoration of crucial gut microbiota. These metabolites are metabolised by colonocytes as their main energy substrate, contribute to energy metabolism of the host, alongside other modulatory roles. It could be hypothesized that an enhanced energy balance via greater SCFA production could help endurance performance.

Evidence suggests microbiota can contribute to endurance exercise capacity through the production of SCFA as an energy source for the host (Okamoto et al., 2019). Although colonocytes get most of their energy substrate from SCFA, they prefer butyrate over acetate and propionate, allowing for some absorption of SCFA and transport to various tissues to be metabolised (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). Antibiotics, as well as a diet low in indigestible carbohydrates, were both shown to negatively influence the composition and function capabilities of gut microbiota, resulting in significant reduction in SCFA production. Reduction in SCFA was associated with a reduced endurance capacity and poorer performance (Okamoto et al., 2019). Acetate has been identified as one of the more important SCFA in terms of energy metabolism, with antibiotic treated mice showing improved endurance performance following acetate infusion (Okamoto et al., 2019). *Veillonella atypica* isolated from human athletes was fed to mice. Lactate generated by intense exercise was metabolised by *Veillonella atypica* in the gut and propionate produced, absorbed into the circulation, and resulted in an augmented run time performance (Scheiman et al., 2019). Hence this link highlights the possibility of SCFA to be used as energy substrate, and the importance of acquiring adequate gut flora to ensure adequate SCFA production.

Probiotic supplementation has been shown to increase serum glucose levels during exercise, as well as increase muscle and liver glycogen storage prior to exercise, thereby reducing physical fatigue (Hsu et al., 2018; Huang, Lee, et al., 2019; Lee et al., 2019). It has been speculated that increased blood glucose during exercise indicates the muscle is taking up less glucose from the blood, possibly due to abundant muscle glycogen stores, or since another energy substrate is being utilised (Huang, Lee, et al., 2019). Additionally, SCFA may modulate hepatic lipid and glucose homeostasis by acting as signalling molecules (den Besten et al., 2015; Huang, Chen, Chuang, Chiu, & Huang, 2019). SCFA production, has also been speculated as a mechanism behind improved glucose uptake for fuel during exercise via upregulation of the GLUT4 transporter (Huang, Lee, et al., 2019). This effect has been shown to improve glucose homeostasis, crucial to exercise performance. Thus, the possibility of probiotic supplementation enhancing SCFA production and improving energy metabolism and other modulatory roles to benefit exercise performance is an exciting area for further research.

Butyrate resulting from anaerobic fermentation, can influence tight junction proteins (Peng, Li, Green, Holzman, & Lin, 2009; H. B. Wang, Wang, Wang, Wan, & Liu, 2012). Upregulated tight junction proteins could improve gut barrier integrity and lessen the risk of GIT disorders during exercise. Butyrate has the ability to increase AMPK enzyme activity, which leads to enhanced reorganization of tight junction proteins, thereby improving the structural integrity of the intestinal barrier (Peng et al., 2009). This link between SCFA and gut barrier integrity

highlights the interplay between proposed mechanisms for probiotic administration, gut microbiota and athletic performance.

2.4.5 Reduced Fatigue

Physical fatigue is defined as a reduction in maximal voluntary muscular power or strength output (Gandevia, 2001). Exercise-induced metabolites such as lactate, ammonia, and blood urea nitrogen (BUN) increase during exercise and can impact on athletic performance. It is speculated that probiotic supplementation may attenuate the production of these metabolites which underlies the basis of one of the possible mechanisms for therapeutic use of probiotic supplementation in sport and exercise.

Excess ammonia can cause fatigue during exercise as this metabolite can interact with the CNS. With intense exercise, high levels of ammonia in the blood are produced via deamination of adenosine monophosphate (AMP) and BCAA which lead to increased uptake of ammonia in the brain (Wilkinson, Smeeton, & Watt, 2010). This results in impaired neural function in the CNS which plays an important role in the onset of physical fatigue by controlling physical coordination and other neural activity crucial to exercise (Finsterer, 2012). Certain probiotic strains have been demonstrated to assimilate ammonia which highlights a possible interaction between probiotics and exercise-induced ammonia accumulation (Deguchi, Makino, Iwabuchi, Watanuki, & Yamashita, 1993). Hence, it has been suggested that probiotic supplementation could contribute to the amelioration of perturbed biochemistry in the central nervous system thought to induce physical fatigue.

During intense exercise, lactate alongside H^+ ions, are produced as by-products of glycolysis (Lee et al., 2019). It has been believed previously that lactic acid reduced pH of blood and muscle tissue, thereby negatively impacting metabolic activity and muscle contraction during exercise. However, more recent developments have suggested that lactate accumulation may play a beneficial role in metabolic processes, acting as a marker of fatigue rather than a direct cause as previously thought (Todd, 2014). Lactate is a key energy source during times of demand, as well as working to restore blood pH. Certain types of gut bacteria have the capacity to use lactate for butyrate production (Hsu et al., 2018; Lee et al., 2019). Evidence from animal models have shown an association between probiotic supplementation and reduced exercise-induced serum lactate levels during physical activity (Chen et al., 2016; Huang, Lee, et al., 2019; Lee et al., 2019). Thus, it is possible that probiotics could enhance capacity of gut bacteria to utilize exercise-induced lactate, thereby increasing the production of SCFA which may act as additional energy substrate and aid exercise performance. The interplay between fatigue related biomarkers, gut microbiota and subsequent SFCA production is an example of how these proposed mechanisms may work in combination to induce beneficial outcomes for performance such as resistance to fatigue and improved energy balance.

BUN is a measure of serum nitrogen levels produced from breakdown of urea, which can reflect protein degradation (Hsu et al., 2018). Disruption to the metabolic activity in the muscle tissue can cause adverse effects on contractile activity and may contribute to muscular fatigue. BUN levels following prolonged exercise are associated with the level of exercise tolerance of the subject and hence fatigue development (Huang et al., 2018). Kefir and single strain probiotic supplementation have been found to reduce BUN levels (Hsu et al., 2018; Lee

et al., 2019). Therefore, probiotic supplementation could help minimise exercise induced fatigue, a concept that would be very important for athletes competing in back to back events.

Overall, it may be a combination of several of these fatigue-associated indices which influence athletic performance. The proposed anti-fatigue effect conferred from probiotic supplementation is an exciting area for further investigation.

2.4.6 Immune modulation and Inflammation

An extensive amount of research has investigated immune modulation and reduced incidence of URTI as the potential benefit of probiotic supplementation for athletes (Pyne et al., 2015). Probiotics have been demonstrated to interact with the commensal bacteria and the host immune system to confer immunomodulatory effects further than the GIT (Jäger et al., 2019). Improvement to respiratory health is thought to occur through modulation of the common mucosal immune system, which makes probiotic supplementation an attractive therapeutic option. Interestingly, it has been theorised that an increase in inflammatory markers in athletes may be due to an exercise-induced reduction in intestinal barrier integrity (Lamprecht & Frauwallner, 2012). Furthermore, it has been hypothesised that changes to immune regulation and subsequent alterations in cytokine levels may start with diversification of gut microbiota which influence a variety of immune cells and their receptors in the gut (Corthésy, Gaskins, & Mercenier, 2007). Probiotic supplementation can result in a significant reduction in potentially pathogenic bacteria situated in the nasal cavity which implies a link between gut diversification and immune benefits (Gluck & Gebbers, 2003). This concept can be referred to as immune cell trafficking, with changes to one mucosal area, for example the gut, affecting a different mucosa, such as the nasal cavity, within the body. This highlights the possibility that these proposed mechanisms may be interconnected, and the complexities are yet to be determined.

Athletes are an at-risk population for URTI due to the oxidative stress and inflammatory responses induced by strenuous exercise and high training loads (Jäger et al., 2019; West et al., 2011). Disturbances to immunity can be induced by exercise and allow an opportunity for infection to take hold. Respiratory tract infections (RTI) can impact athlete's ability to train and compete, thereby negatively impacting performance. Thus, probiotic supplementation has been proposed as a tool to reduce the incidence and symptoms of RTI, allowing greater adherence to training schedules, which could indirectly benefit athletic performance (Salarkia et al., 2013; Strasser et al., 2016). As an example, although the reduction in RTI did not directly influence performance, the improved respiratory health status may have contributed to the improved VO_2max observed in the probiotic group in the study by Salarkia et al. (2013).

Additionally, it has been reported that the reduction in URTI incidence is likely due to the lactic acid bacteria-induced activation of dendritic cells (pDC) (Komano et al., 2018). pDC are antigen presenting cells that initiate adaptive immune response to viruses through production of interferons. Moreover, it has been speculated that probiotics could modulate the mucosal immune system at the intestinal level via pattern recognition receptors such as toll-like receptor 9 (TLR9) pathway following ligand binding by metabolites of gram-positive bacteria, such as Lactobacillus and Bifidobacterium strains (Wells et al., 2011). *Lactococcus lactis* JCM 5805 acts as an agonist ligand binding to toll-like receptor 9 (TLR) (Jounai et al., 2012). This specific TLR acts as an important receptor as part of an immune response to initiate signalling cascades (Komano et al., 2018). This enhanced antiviral capacity is the

reason authors believed URTI symptoms can be significantly reduced through lactic acid bacteria supplementation, such as *Lactococcus lactis* JCM 5805.

Inflammatory responses can be augmented following intense exercise, including alterations in cytokine levels (Pedersen & Toft, 2000). Probiotic supplementation may act to reduce pro inflammatory cytokine production while increasing levels of anti-inflammatory cytokines directly after exercise (Huang, Wei, et al., 2019). In support of this mechanism, resting interleukin-6 (IL-6) levels have been found to be suppressed after supplementation with probiotics compared to placebo group which observed elevated IL-6 concentration (Jäger, Purpura, et al., 2016). IL-6 is a potent pro-inflammatory cytokine (Tanaka, Narazaki, & Kishimoto, 2014).

Levels of C-reactive protein (CRP), a marker of inflammation, have been shown to be suppressed during strenuous exercise in athletes on kefir supplementation (O'Brien et al., 2015) and following probiotic yoghurt supplementation (Salehzadeh, 2015) which could reflect beneficial effects of probiotic supplementation on inflammatory modulation. Probiotic supplementation has been proposed to supress increases in tumour necrosis factor alpha (TNF-α) induced by aerobic exercise and resistance type exercise (Townsend et al., 2018; West et al., 2011). Thus, it could be postulated that probiotic-induced proinflammatory cytokine suppression reduces inflammation and may indirectly improve performance through better health status and greater training capacity. Another possible benefit of probiotic supplementation and the link to reduced inflammation is the possibility of improved muscle mass since cytokines can initiate atrophy of muscle (Chen et al., 2016). Chen et al. (2016) reported increased muscle mass in mice with probiotics and no training intervention. It was speculated that the mechanism behind these changes could be linked to reduced inflammatory markers, presumably caused by probiotic supplementation. Authors of a substantial review have reasoned that pro-inflammatory cytokines, such as IL-6 and TNF-α, contribute to muscle atrophy, therefore their attenuation following probiotic supplementation may allow for an enhanced muscular response (Costa, Snipe, Kitic, & Gibson, 2017; Costamagna, Costelli, Sampaolesi, & Penna, 2015).

Table 3. Studies with evidence to support certain proposed mechanisms of actions for probiotic supplementation and aerobic performance

	Gut microbiota diversification	Gut permeability and discomfort	Inflammation/ Immune modulation	SCFA	Protein utilisation	Fatigue associated markers
(Carbuhn, 2017)			✓			
(Carbuhn et al., 2018)	✓					
(Chaves et al., 2018)	✓	✓				
(Chen et al., 2016)			✓	✓	✓	✓
(Cox et al., 2010)			✓			
(Desbonnet et al., 2008)			✓			
(Hsu et al., 2018)	✓			✓		✓
(Huang, Lee, et al., 2019)				✓		✓
(Huang, Wei, et al., 2019)			✓			✓
(Huang, Pan, et al., 2020)	✓	✓	✓	✓		
(Huang, Hsu, et al., 2020)	✓		✓			
(Ibrahim, Muhamad, Ooi, Meor-Osman, & Chen, 2018)					✓	
(Jäger, Purpura,			✓			

et al., 2016)						
(Jäger, Shields, et al., 2016)					✓	
(Kekkonen et al., 2007)		✓				
(Lamprecht et al., 2012)		✓	✓			
(Lee et al., 2019)				✓		✓
(Lin et al., 2020)	✓			✓		
(O'Brien et al., 2015)			✓			
(Salarkia et al., 2013)			✓			
(Salehzadeh, 2015)			✓			
(Sashihara et al., 2013)			✓			
(Sawada et al., 2019)	✓					
(Shing et al., 2014)		✓	✓			
(Soares et al., 2019)	✓		✓	✓		
(Strasser et al., 2016)			✓			
(Toohey et al., 2018)		✓			✓	
(Townsend et al., 2018)	✓		✓			
(West et al., 2011)			✓			

2.5 Heat stress

Aerobic exercise challenges the homeostasis of the body. To support elevations in metabolic activity and oxygen consumption induced by an increase in muscle contractions, the thermoregulatory, cardiovascular and respiratory systems adjust by increasing skin blood flow, sweat rate, HR and breathing rate to maintain homeostasis. Hot environmental conditions cause an additional challenge to the body, and therefore exercise performance (Cheuvront, Kenefick, Montain, & Sawka, 2010). With hot environmental conditions the sweat response is increased since heat loss by evaporation of sweat is usually the main thermoregulatory response under these conditions. However, there is a finite capacity for thermoregulation and eventually increasing thermal and cardiovascular strain will impair aerobic performance and increase risk of developing heat illness. Therefore, environmental heat stress presents an additional challenge to the athlete during aerobic activity.

Further, the combination of hot temperatures and aerobic exercise can impact exercise performance through other physiological perturbations (Costa et al., 2017). GIT discomfort and respiratory tract illness are common complaints for endurance athletes training and competing under warm or hot conditions. While gut permeability has been shown to increase with endurance type exercise (Nieman, 1997), it appears heat stress can further augment the negative effect on intestinal permeability induced by exercise stress (Shing et al., 2014). It could be assumed that heat stress amplifies gut barrier dysfunction induced by exercise, however there is evidence to support disruptions to intestinal integrity caused by heat stress alone. Ogden et al. (2020) reported increases to intestinal fatty acid binding protein (I-FABP), a key gut injury marker, following 2 x 40-minute bouts of 6 km/hr walking at 7% gradient in warm conditions (35°C, 30% relative humidity (RH)). However, greater exercise intensity paired with warm conditions is thought to likely increase the perturbations to the gut. Hyperthermia may cause reduction in the integrity of the gut barrier and increase intestinal permeability, as seen in *in vitro* and animal model examples (Koch et al., 2019; Lambert et al., 2002; Oliver et al., 2012). The molecular mechanism underlying this process remains unclear, although there are discussions around the role of oxidative stress and thermal damage directly to the intestinal epithelial cells during hot environmental conditions (Lambert et al., 2002).

Immune function is also significantly altered in athletes with high training loads (Nieman & Wentz, 2019). With the addition of an adverse environment, such as heat stress, athlete's impaired immune functioning can be further exacerbated. An early review concluded that the additive effects of strenuous exercise together with an environmental stressor, like heat, is likely to induce an immunosuppressive response, whereby pro inflammatory cytokines are released as an early response to core temperatures increasing between 2-4 °C over 60 minutes (Shephard, 1998). However, it is important to note the interaction between exercise and immune function is complex, with low – moderate exercise thought to improve immune responses and high training loads and additional stressors linked to immune dysregulation and increased risk of illness (Nieman & Wentz, 2019).

Heat stress can act to augment these immune and physiological gut responses, increasing the risk of GIT illness, respiratory tract illness and inflammation for those exercising in warm - hot temperatures. While details behind these mechanisms are not fully understood, there

is definite cause for further investigation into the application of probiotic supplementation as a tool to aid exercise- induced ailments during exercise and possibly improve performance outcomes. In addition to stressing the body, global warming and more competitions being held in warm climates, it will become more common to have athletes exposed to heat. Thus, research is required to investigate probiotic supplementations as a possible therapeutic aid to help athlete's exercise performance. Furthermore, future researchers need to ensure that the appropriate heat stress protocol is implemented in order to exacerbate any changes to gut function, permeability, and immune and inflammatory responses to enhance the likelihood of seeing a benefit following probiotic supplementation.

In addition to the performance outcomes of runners discussed above, two researchers have used both heat and exertional stress to investigate the effect of probiotic supplementation on running exercise. Of the two studies, one reported a positive effect on running performance in hot environmental conditions (Shing et al., 2014), whereas the other study failed to show any benefit to performance (S. K. Gill et al., 2016). A randomised crossover trial Shing et al. (2014) found that ten trained male runners showed a significant increase in run-to-fatigue time under heat stress conditions with probiotic supplementation. Athletes were provided daily capsules containing 4.5 x 10^{10} CFU of a multistrain probiotic containing nine *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains for 4 weeks. Conditions for the exhaustive treadmill test included 80% VT at 35˚C and 40% RH. Interestingly, HR, ratings of perceived exertion and core temperature were not different between trial groups. Despite low sample size (n=10), authors provided a robust a priori sample size estimation, and whilst their results for lipopolysaccharide (LPS) and dual sugar test may be underpowered, their reported performance effect is real.

In contrast, a study by S. K. Gill et al. (2016) failed to demonstrate any beneficial effects of 1 x 10^{11} CFU/day *Lacticaseibacillus casei* supplementation on physiological measures of trained endurance athletes in the heat. Eight male athletes were subjected to 2 hours of sub maximal running (60% VO_2max) at a steady speed determined using the VO_2max-work rate relationship. This session took place in a controlled environmental chamber, set at 34˚C and 32% RH. Like Shing et al. (2014), no significant changes in HR or rating of perceived exertion were found between the two groups. Additional measures of rectal temperature and thermal comfort rating did not differ between probiotic or placebo groups either. No direct performance test was conducted. It is possible that the supplementation protocol involving a high daily dose of single strain probiotic for 7 days was an insufficient intervention period to exert benefit to physiological responses such as HR and core temperature. However, admittedly, Shing et al. (2014) used a lower dose of multistrain probiotic during an intervention period four times as long as Gill et al. (2016) yet was still unable to show significant changes to HR or core temperature. Other authors who have reported improvements in endurance exercise (notably in thermoneutral environmental conditions) have implemented a supplementation period of at least 4 weeks, thus a longer intervention period may be required for benefits to endurance athletes under heat stress.

Alongside the theory that the short supplementation period used in the study by S. K. Gill et al. (2016) may be a contributing factor to the absence of physiological changes observed, several other studies that also failed to report positive performance or physiological

outcomes were implementing suboptimal conditions for their probiotic intervention study designs. Many studies mentioned above did not use a heat stress protocol or ensure exercise intensity and duration was moderate to high, thereby failing to induce sufficient stress on the gut and therefore did not achieve the two critical factors for exercise performance probiotic intervention studies:

1. Sufficient stress on the gut, including indirectly affecting inflammatory response and directly affecting the GIT through heat and/or exercise mode, intensity and duration
2. Optimal supplementation protocol, specifically adequate dose, duration, and strain.

To the authors knowledge, no studies have investigated the potential of Fonterra proprietary probiotic strains - *Lacticaseibacillus rhamnosus* HN001 and *Bifidobacterium animalis* ssp. *lactis* HN019 - to induce performance benefits during exercise in warm environmental conditions.

2.6 Proprietary strains

In the late 1990's, Fonterra Research and Development Centre screened more than 2000 lactic acid bacterial strains in their culture collection for probiotic potential (Prasad, Gill, Smart, & Gopal, 1998). Of these strains, *Lacticaseibacillus rhamnosus* HN001 and *Bifidobacterium animalis* ssp. *lactis* HN019 were further researched due to their ability to survive harsh conditions similar to the GIT, a crucial characteristic for future commercialisation of probiotics. Known commercially as DR 10 and DR 20, these probiotics are of dairy origin and were initially found to exert immune enhancing properties in mice (H. S. Gill, Rutherfurd, Prasad, & Gopal, 2000). More recent work has demonstrated HN001 and HN019 to have several other health-promoting effects including enhanced intestinal barrier integrity, improvement in constipation, protection against respiratory infection, enhanced nutrient absorption and reduced inflammation. To the authors knowledge, no studies have investigated the performance enhancing effects of HN001 or HN019 strains during exercise. However, the beneficial effects exerted by HN001 and HN019 are similar to the mechanisms of action of the performance enhancing probiotic strains, therefore Fonterra's proprietary probiotics could potentially have a positive impact on athletic performance.

2.6.1 *Lacticaseibacillus rhamnosus* HN001

Gut barrier integrity

Gut barrier integrity was shown to be enhanced with HN001 following the administration of 1×10^7 CFU to an intestinal cell model (Anderson, Cookson, McNabb, Kelly, & Roy, 2010). Another *in vitro* study showed similar improvements to structural integrity of the gut barrier in combination with *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* strains and milk carbohydrate (Barnett, Roy, Cookson, & McNabb, 2018). Moreover, this probiotic strain has been shown not to degrade gastric mucin, the key glycoprotein in the mucus layer *in vitro*, thereby can maintain the structure of the protective mucous layer (Zhou, Gopal, & Gill, 2001). The mucus layer acts to protect the underlying epithelial layer in the gut barrier, suggesting further beneficial effects of HN001 on gut barrier integrity.

Nutrient absorption

Mineral absorption, including calcium and magnesium, may be enhanced following HN001 supplementation (Kruger, Fear, Chua, Plimmer, & Schollum, 2009). Ovariectomised rats were given daily HN001 supplementation at 1×10^9 CFU for 12 weeks. The HN001 group showed

attenuated rates of bone loss compared to control group, which indicated HN001 had a positive impact on bone mineral density. Authors speculated that reduced pH from greater SCFA generation, or protective effects against pathogenic flora through enzymatic activity may underlie the mechanisms for beneficial probiotic effects on host mineral absorption.

Mood and depression

HN001 exhibited positive effects on mental state in postpartum women in a New Zealand trial which provided daily supplementation at 6 x 10^9 CFU from 12-14 weeks until 6 months post-partum (Slykerman et al., 2017). Significantly lower depression and anxiety scores were observed following this long term HN001 supplementation.

Immune modulation and infections

Early studies using animal models reported enhanced immune responses following HN001 supplementation, including improvements in IFN-y, antibodies, and natural killer cell activity, as well as reduced severity of infection (H. S. Gill, Shu, Lin, Rutherfurd, & Cross, 2001; Shu & Gill, 2002). Similarly, a clinical study with a middle aged and elderly population found 3 weeks of supplementation at 1 x 10^9 CFU/g HN001 daily, enhanced immune responses (Sheih, Chiang, Wang, Liao, & Gill, 2001). Phagocytic activity and natural killer cell activity were increased which suggests a role for HN001 in enhancing natural immune system in healthy adults. Also, a significant reduction in serum IL-6 levels was reported in HIV patients following long term symbiotic treatment over 16 weeks including 1 x 10^9 CFU/mL of HN001 and another Bifidobacterium strain (González-Hernández et al., 2012). IL-6 is a pro inflammatory cytokine that has been linked to chronic inflammatory diseases (Gabay, 2006).

Additionally, four weeks of 1 x 10^{10} CFU/day of HN001 has been shown to reduce *Staphylococcus aureus* carriage in the gut (Eggers et al., 2018). This bacterial pathogen can increase the likelihood of other infections. Probiotic supplementation may decolonize *Streptococcus aureus* and contribute to lowering the risk for infection. A combination of 1 x 10^9 CFU/day of HN001 with a *Lactobacillus acidophilus* strain for four weeks may help to significantly reduce the number of *Clostridium difficule* in the intestine of elderly who harboured levels of this bacteria at baseline (Lahtinen et al., 2012). HN001 has been demonstrated to be involved in immune response against gastrointestinal infection in infants (Good et al., 2014). It appears HN001 immune response effects are mediated via TLR9 which can sense foreign DNA of some pathogens to induce an innate immune response and protect the host from pathogenic invasion. While HN001 exhibits beneficial immunomodulatory effects, evidence indicate that HN001 supplementation in an animal model does not induce a severe inflammation state (Zhou & Gill, 2005).

2.6.2 *Bifidobacterium anamalis* ssp. *lactis* HN019

Gut microbiota

Alterations in gut microbiota composition have been observed in thirty healthy adults aged 20 – 60 years following four weeks of HN019 supplementation at 1 x 10^{10} CFU/day (Gopal & Gill, 2003). Increases were seen in Bifidobacterium and lactobacillus strains in the gut, which are bacterial genera with links to health promoting benefits. Similarly, intestinal microflora of elderly adults was altered with HN019, including increases in *Lactobacillus*, *Bifidobacteria* and *Enterococcus* phyla amounts (Ahmed, Prasad, Gill, Stevenson, & Gopal, 2007). Reductions in enterobacteria counts were reported which is important as this bacterium can be detrimental

to health and cause intestinal upset. These changes were observed even for the low dose protocol of 6.5 x 10^7 CFU/day over four weeks.

Gut discomfort

HN019 has been shown to relieve symptoms of constipation by improving bowel movement frequency and decreasing whole gut transit time (Ibarra, Latreille-Barbier, Donazzolo, Pelletier, & Ouwehand, 2018; Waller et al., 2011). Both studies reported HN019 to be well tolerated without any adverse events during the 2-3-week intervention periods using similar dosage protocols, approximately 1 x 10^{10} CFU/day. A synbiotic combination of both 1 x 10^8-10^9 CFU HN019 and HN001 over 30 days, together with fructoligosaccharide improved stool frequency, consistency and constipation intensity in chronically constipated women (Waitzberg et al., 2013).

Immune modulation and infections

It is accepted that gut microflora composition alters with age, which could contribute to the suppressed immune responses observed in both young and elderly populations making them more susceptible to infections (Clemente, Ursell, Parfrey, & Knight, 2012; Clements & R. Carding, 2018). HN019 has been demonstrated to enhance immune cell activity in healthy elderly adults according to a review of four clinical trials with a dose range of 5 x 10^9 to 3 x 10^{11} CFU over 3 - 6 weeks (Miller, Lehtoranta, & Lehtinen, 2017). Additionally, significantly higher phagocytic activity, as well as increased specific antibody responses, have been documented in an animal model (H. S. Gill et al., 2000). Enhanced phagocytic capacity and natural killer cell activity may provide greater resistance against infections. Furthermore, evidence suggests 9 months of 2-5 x 10^9 CFU/day HN019 offers protection against diarrhoea incidence in children, which is another population with reduced immune response (Hemalatha et al., 2014). Together with a prebiotic in a fortified milk vehicle, long term HN019 supplementation at 1.9 x 10^7 CFU/day for one year has also shown to significantly reduce the incidence of pneumonia and lower respiratory illness in children (Sazawal et al., 2010). Notably, while HN019 appears to stimulate immune responses to protect against infection, possibly by mediated effects on microbial balance, improved gut health and subsequent absorption of key nutrients, this probiotic does not induce pathological inflammation in an animal model (Zhou & Gill, 2005).

Overall, the established health promoting benefits of Fonterra's proprietary strains may offer an indication as to whether these strains might confer benefit to athletic performance. For example, it appears much of the research on HN001 and HN019 in human health has been in the area of immune and inflammatory responses, predominantly influencing gut and respiratory tract health. There is a possibility this relationship might underlie benefit in sport performance. The HN019 induced increases in immune cell activity are likely to contribute to improved resistance against pathogens and help to reduce incidence of infections. Similarly, athletes can present with vulnerability to infectious agents due to the exercise-induced stress suppressed immune response and travelling-induced diarrhoea incidences. Improved resistance against infections, via enhanced immune cell activity and improved intestinal barrier integrity, may prove beneficial for athletic performance. The previous studies investigating the roles of HN001 and HN019 in immune, gut, and mental health was done in different populations, therefore this data cannot be extrapolated to healthy and active adults. Therefore, although it appears Fonterra's proprietary strains may help enhance immune or

gut health which in turn could play a role in improving performance of athletes, it remains to be proven.

2.7 Conclusion

This review of the literature on probiotic supplementation in sport and exercise performance found mixed results regarding the efficacy of probiotic supplementation as an ergogenic aid for various exercise modes. The inconclusive findings highlight the highly strain specific effects conferred by probiotic ingestion, together with the impact of various methodologies used in the 30 studies identified in this review. Despite a seemingly adequate number of studies, evidence of beneficial effects of probiotic supplementation on exercise performance remains weak. Thus, currently it cannot be concluded that probiotics, on the whole, act as ergogenic aids to enhance athletic performance. Although performance benefits remain ambiguous, several different mechanisms of action for ergogenic effect with probiotic supplementation were postulated from these studies. Seven common mechanisms were identified including gut microbiota diversification, intestinal permeability, AA absorption and adaptation to exercise, SCFA production, reduced fatigue, central mechanisms and mood, and immune modulation and inflammation.

Nonetheless, some bacterial strains did demonstrate beneficial effects on exercise performance. The mixed results are likely due to the strain specific effects and mechanisms of action. Different strains, although from the same bacterial species, may not be as effective or even confer the same benefit to the host. Hence, performance outcomes will vary between bacterial strains. Fonterra's proprietary strains have proven health benefits in several populations other than athletes. Interestingly, several of these health benefits have similarities to some of the mechanisms of action proposed to exert benefits to performance. This suggests some potential for HN001 and HN019 to also exert ergogenic effects. However, until further work is conducted, any similarities to mechanism of action between Fonterra's proprietary strains and strains with ergogenic benefit are purely speculative. Further research is required to investigate the use of HN001 and HN019 combined as an ergogenic aid in the active and sporting population. Moreover, additional research will be necessary for greater understanding of the mechanism of action for any ergogenic effects from these proprietary probiotics.

Chapter Three: Research Aim and Hypotheses

The research conducted in this thesis has been underpinned by the undertaking of a comprehensive literature review, as seen in **Chapter Two**. From this review it is apparent that further research into the efficacy of probiotics as a performance enhancing aid during sport and exercise is required.

The aim of this study was to investigate if 4 weeks supplementation of dual probiotic strains, *Lacticaseibacillus rhamnosus* HN001 and *Bifidobacterium anamalis* ssp. *lactis* HN019, directly affect performance outcomes during a 15-min time trial run in the heat (30°C, 50% RH). A secondary aim was to investigate the effect of probiotic supplementation on physiological measures during the 15-min time trial run in the heat.

The primary hypothesis for the current study is outlined below:

1. 4 weeks supplementation of probiotic will improve running performance in the heat (30°C, 50% RH) as determined by distance (km) completed during 15-min time trial

Additional to the main hypothesis, secondary hypothesis includes:

2. 4 weeks supplementation of probiotic will improve physiological measures, including HR and core temperature, during 15-min time trial in the heat

Chapter Four: Methods

4.1 Experimental overview

Seven recreationally trained male runners completed a 12-week study protocol. This consisted of a double-blinded, crossover design with 4 weeks of randomised probiotic or placebo supplementation followed by a 1 hour running trial in a heat chamber (30°C, 50% RH) at the end of each supplementation period. The trial involved a 45-min pre-load where participants ran on a treadmill at 70% of their VT followed immediately by a 15-min self-paced time trial. Participants completed a 3-week washout period in between the two supplementation periods. Figure 1 provides a diagram of the experimental overview.

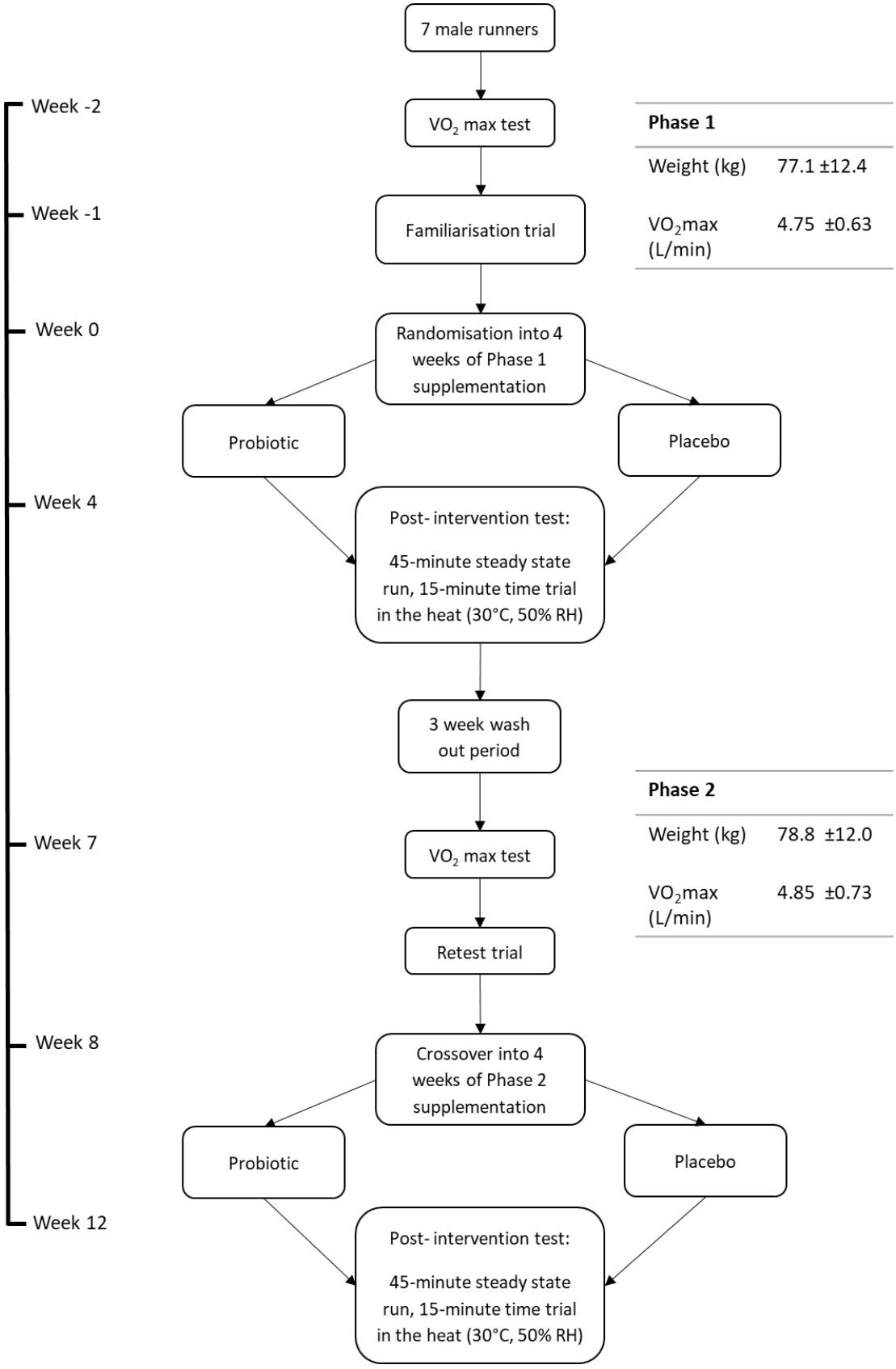

Figure 1. Overview of experimental study design

4.2 Participants

Seven healthy, male athletes were recruited for this study. All participants were comfortable running for 60-min, had a VO_2max >45 mL/kg/min, were not heat acclimated and were not smokers. Participants had not consumed antibiotics or certain probiotic dietary products in the three weeks prior to and throughout the study period. The participants were recreationally trained for endurance running, although several also played team sports such as football. Participants characteristics are displayed in Table 4. Participants were randomly assigned to the first intervention group and completed the remaining intervention during the second phase of the trial. The randomisation was completed by a third party and unblinding occurred following the completion of data collection. All participants were fully informed about the experimental protocol and possible risks before providing written informed consent prior to commencing the trial. This protocol was approved by the Massey University Human Ethics Committee (MUHEC SOA 19/73).

Table 4. Participant characteristics on entry to study (n=7)

Characteristics	Probiotic
Age (years)	31 ± 8
Height (cm)	176.9 ± 10.4
Ventilatory threshold (VT) (km/hr)	16.1 ± 0.6
Treadmill speed (km/hr) @ VT	11.1 ± 0.5

Values are means ± SD. Treadmill speed refers to approximately 70% of VT

4.3 VO_2max test

Participants completed an incremental test to determine VO_2max as part of the screening process. Participants completed the test in the morning on a treadmill (TRUE, MO, USA) in a moderate environment (19°C, 50% RH). The test protocol used was based on a previous protocol by Shing and colleagues (Shing et al., 2014). Participants commenced the test at 10 km/hr, 0% gradient. Every minute thereafter the speed would increase by 1 km/hr until the speed 18 km/hr. Following 1 minute at 18 km/hr, the gradient increased by 1% per minute. Participants finished the test once they reached exhaustion. HR (Polar, Polar Electro Oy, Finland) and RPE (Borg, 1998) measures were taken every minute during the test. Expired gas was measured continuously for the duration of the test, analysed (AEI Technologies, PA, USA) and displayed (TurboFit, VacuMed, Ventura, CA, USA) as 15-s averages. A plateau in VO_2max despite increased workload, age-predicted maximal HR and an RER>1 confirmed VO_2max.

4.4 Ventilatory threshold

Each individual's VT was determined by plotting the participant's ventilation (L/min) against their speed (km/hr) during their VO_2max test, with a departure from linearity determining the VT. The speed at which participants were to complete the 45-min preload stage of the experimental running trial was based on 70% of their individual VT, whereby participants ran at this speed for 15-min following their VO_2max test as confirmation.
70% VT = Speed (km/hr) at VT x 0.7.

4.5 Nutrition intervention

Following a familiarisation trial, participants commenced daily supplementation of one capsule for 4 weeks. Participants were randomised into the first phase supplementation and crossed over into the other supplementation group to complete the second arm of the trial following 3-week wash out period. Each intervention supplement capsule contained 6 x10^9 CFU *Bifidobacterium animalis* ssp*. lactis* HN019 and 6 x 10^9 CFU *Lacticaseibacillus rhamnosus* HN001. These two probiotic strains are proprietary to Fonterra Co-Operative Limited and have been previously proven to survive the harsh conditions similar to gastrointestinal environment and establish in the microbiome of the colon (Prasad et al., 1998).

The strains were provided by Fonterra Research and Development Centre (Palmerston North, New Zealand). The placebo capsules contained maltodextrin. Both sets of capsules were visually identical and were kept refrigerated. Participants were asked to return any unused pills at the end of the 4-week supplementation period. This was to assess compliance during the supplementation period.

4.6 Standardisation

4.6.1 Diet and lifestyle

A 48-hour estimated food diary was completed prior to the experimental trial. The diet record was analysed using Foodworks software (Version 10, Xyris software, Brisbane, Australia) which utilises FOODfiles 2016, New Zealand's Food Composition Database. Participants were asked to replicate, as close as possible, their diet during the second arm of the study based on the 48-hour recorded period prior to the Phase 1 experimental testing session. By replicating and obtaining similar daily energy intake and macronutrient intake in the 48 hours leading up to both trial session, variability of performance within subjects due to metabolic profile can be minimised. Participants were asked to consume their standard pre-race breakfast at least 2 hours prior to their testing session on the morning of their experimental trial. They consumed the same individualised pre-trial meal for both trials, typically consisting of a small, carbohydrate-based snack and hot beverage. Participants avoided certain fermented and probiotic dairy products, including Whitestone Company cheeses, Mainland cheeses, fermented probiotic dairy drinks, Symbio and Activia products for three weeks prior and throughout the duration of the study period.

4.6.2 Exercise

Participants were asked to complete a 48-hour physical activity diary prior to their experimental trial. This physical activity record was modified from Bouchard and colleagues' 3-day activity record used to assess energy expenditure in children and adults (Bouchard et al., 1983). Over a 48-hour period prior to the experimental trial, participants were asked to fill in their physical activity in 15-min periods. Each 15-min period of activity was assigned a categorical value (0-9) that reflected the exercise intensity which corresponded to a range of METS and kcal/kg/15 min. The two days of physical activity prior to the Phase 1 trial were replicated as best as possible for the second arm of the study to minimise variation in glycogen stores and subsequent performance during the experimental trial. Participants were also asked to refrain from any competition racing in the week prior and to avoid strenuous physical activity in the 24 hours prior to the trial.

4.6.3 Hydration

Participants provided a midstream urine sample prior to the performance test which was assessed for hydration status using a refractometer (Master URC NM, Atago, John Morris Scientific Ltd, Sydney, AUS). Euhydration was indicated by USG of < 1.020 (Sawka et al., 2007). During the main experimental trials, participants were provided with 1.5 mL/kg of tap water every 15 minutes. This water rate was provided to reduce the risk of participants becoming dehydrated during the 1 hour run in the heat. Amount of water consumed was recorded. Changes in body mass (kg) due to fluid loss were also recorded. An assumption of 1 L water is equal to 1 kg was made.

4.7 Performance test

The night prior to the performance test participants ingested a radio temperature pill before going to sleep (CorTemp, HQ Inc, Palmetto, FL, USA). On arrival at the lab the next morning, participants were asked to void their bladder and provide a mid-stream urine sample. Both trials occurred at the same time of the day (± 1 hour), usually 8-9am. Participants were asked to avoid non-steroidal anti-inflammatory drugs (NSAIDS) on the day of their trial up until their 48-hour blood sample was completed. Participants completed a one hour run on a treadmill (TRUE, MO, USA) in the heat chamber with conditions set at 30°C, 40-50% RH (average temperature 29.4°C, average RH 54.2%). The run protocol involved 45-min run at 70% of individual VT, followed by 15-min self-paced time-trial. Participants were able to view the distance completed; however, pace or time was not displayed to the participants. The researcher provided updates of every 5 minutes elapsed and time remaining throughout the trial. No external encouragement was provided during the trial. Participants were instructed to do what they need to complete the time-trial in the fastest time possible. A fan placed approximately 1 metre in front of the participant on the floor was positioned towards their lower body and set at medium intensity (12 km/hr) during the 45-min preload stage and switched to the highest intensity (15.5 km/hr) during the time trial.

Distance completed (km) during the 15-min time trial was the main performance measure recorded for the current thesis. HR (Polar, Polar Electro Oy, Finland) and core temperature (CorTemp, HQ Inc, Palmetto, FL, USA) was recorded every 5 minutes during the run. RPE (Borg, 1998) was recorded every 15 minutes. Every 15 minutes during the first 45 minutes, an expired gas sample was taken for 2 minutes. At the end of the 45-min preload stage and at the completion of the time trial, gastrointestinal discomfort was assessed using a 10-question modified version of the gastrointestinal symptom rating scale (GSRS) (Revicki, Wood, Wiklund, & Crawley, 1998). Body weight (wearing sports shorts only) was measured at the end of the trial after participants towelled off any moisture from their skin.

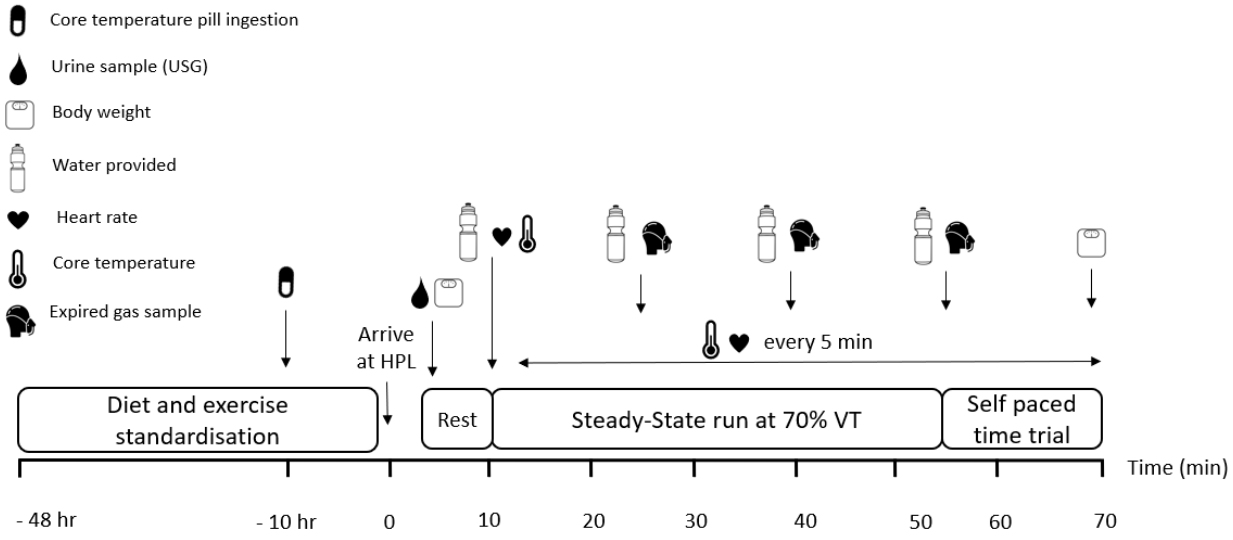

Figure 2. Timeline of experimental procedure during day of trial

4.8 Data analysis

Fluid loss (kg) = Pre exercise weight (kg) + water consumed during exercise (L) - post exercise weight (kg)

4.9 Statistical analysis

Data was assessed for normality using a Shapiro Wilk test. A Wilcoxon Signed Rank test was used for fluid intake during the trial and average daily water intake. Paired *t*-tests were used to compare baseline values between probiotic and placebo trials. A two-way repeated measure analysis of variance (ANOVA) test was used to assess changes to HR and core temperature over time between probiotic and placebo trials. All data was assessed for sphericity. Test-retest reliability was assessed using coefficient of variation (CV), calculated as CV% = standard deviation / mean * 100

Data was analysed using IBM SPSS statistical software (Version 26, Chicago, IL, USA). Statistical significance was set at $p < 0.05$. All data was reported as mean ± standard deviation (SD) for 7 participants, unless stated otherwise.

Chapter Five: Results

5.1 Standardisation

From the self-reported physical activity record, total daily energy expenditure (MET/day) was 15,362 METs/day and 15,048 METs/day for probiotic and placebo groups, respectively. As seen in Table 5, the 48-hour period of physical activity prior to each trial that can be described as intentional exercise (defined by >3.3 METs/15 min) and can influence glycogen stores was not different (p=0.65).

As shown in Table 5, daily water intake and macronutrient breakdown was not different between groups in the 48-hour period leading up to the trial.

Table 5. Total energy balance, macronutrients breakdown and hydration status during 48-hour standardised diet period prior to both trials

Trial	Probiotic	Placebo	p-value
Daily energy intake (kJ/day)	8374.3	9052.6	0.31
Macronutrient breakdown (g/kg/day)			
CHO	2.3 ± 1.1	2.5 ± 1.2	0.06
Fat	1.2 ± 0.4	1.4 ± 0.6	0.61
Protein	1.3 ± 0.5	1.4 ± 0.6	0.33
Intentional energy expenditure (METs/day) [+]	6264.8	5688.5	0.65
Water intake (L/day)	3.1 ± 1.4	3.3 ± 1.3	0.50
USG	1.013 ± 0.01	1.012 ± 0.01	0.57

Values are means with ± SD. [+]Intentional energy expenditure based on physical activity that was described by participant to be greater than 3.3 METs/15 min. Examples include light manual work, home exercises, biking/walking to vigorous sports and running.

5.2 Reliability

5.2.1 Time trial performance

From Table 6, it can be seen that running distances completed during the time trial of the familiarisation and first trial were different (p=0.04), while times between trial 1 and trial 2 were not different (p=0.09). Furthermore, reliability of the 15-min time trial performance is improved, as shown in Table 7, following a familiarisation trial. The reliability of physiological variables between trial 2 and 3 includes CV of 2.38%, and 2.49% for HR and VO_2 responses, respectively.

Table 6. Individual performance of running distance complete (km) during 15-min time trial of familiarisation 1, test and retest

Subject	Familiarisation 1	Test (Trial 1)	Retest (Trial 2)
1	3.06	3.26	3.15
2	2.82	2.88	2.76
3	2.86	3.18	2.89
4	3.17	3.01	2.89
5	3.4	3.66	3.00
6	2.46	2.80	2.80
7	2.68	2.90	2.93
Mean	2.92 *	3.14	2.92
SD	0.31	0.30	0.13

Values are means. * indicates significance different to Test (Trial 1)

Table 7. Measure of reliability for running distance complete during 15-min time trial (%)

Measures of reliability	Familiarisation – Trial 1	Trial 1 – Trial 2
Coefficient of variation (CV)	5.29 ± 2.50	4.26 ± 4.81
Lower limit	2.98	- 0.19
Upper limit	7.61	8.71

Standard deviation shown as ±. Lower and upper limits calculated as 95% confidence interval for mean.

5.3 Physiological measures

Figure 3 illustrates an increase in core temperature during both trials from 36.97 ± 0.35°C and 37.02 ± 0.15°C at baseline to 39.55 ± 0.55°C and 39.50 ± 0.44°C at the end of the time trial for the probiotic and placebo groups, respectively. There was no significant effect of trial (p=0.72) for baseline temperatures. There was no main effect for treatment (p=0.89) or interaction (p=0.74), while there was an effect of time (p=0.00).

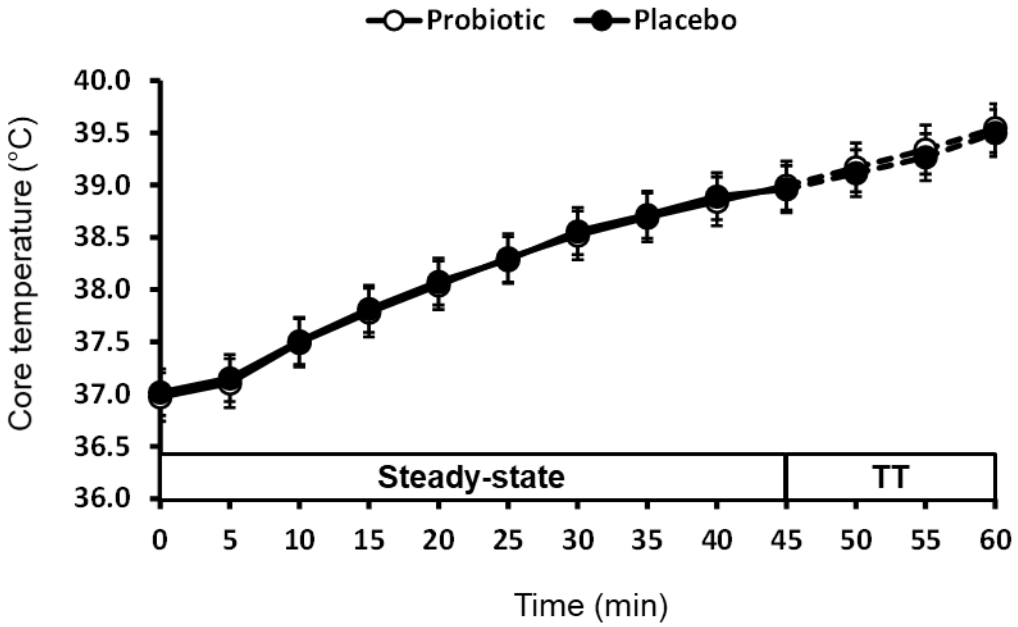

Figure 3. Core temperature during 1 hour running protocol

As seen in Figure 4, the changes in core temperature during the 1 hour run of 2.57 ± 0.49°C and 2.48 ± 0.40°C for the probiotic and placebo groups, respectively, were not statistically different (p=0.75).

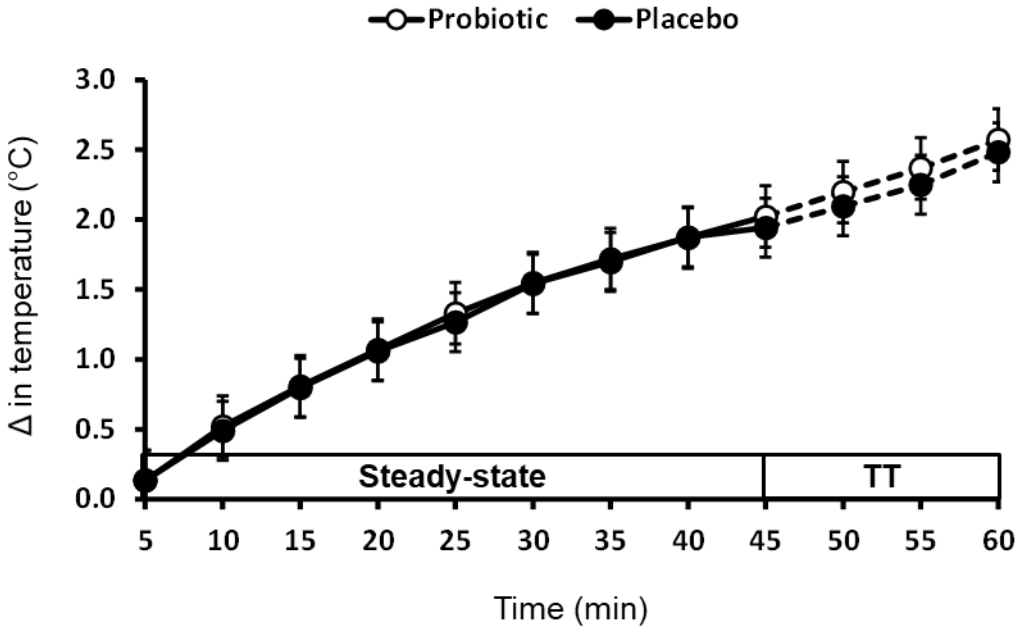

Figure 4. Change in core temperature during 1 hour running protocol

Figure 5 illustrates an increase in HR during both trials from 74 ± 14 bpm and 77 ± 10 bpm at baseline to 186 ± 18 bpm and 189 ± 14 bpm at the end of the time trial for the probiotic and placebo groups, respectively. There was no significant effect of trial on baseline HR (p= 0.53). There were no main effects of treatment (p=0.97) or interaction (p=1.00), while there was an effect of time (p=0.00). Both probiotic and placebo groups were working at 98% HR max, based on peak HR attained during the time trial which was not significantly different

(p=0.73). There was no difference (p=0.87) between groups for average HR max of 85% for both trials during the steady state stage of the protocol.

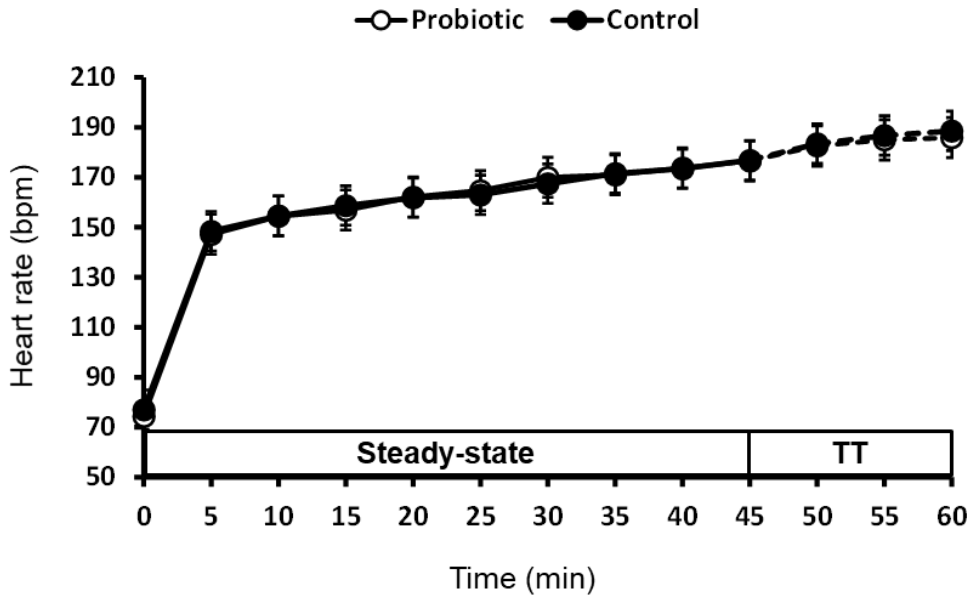

Figure 5. Heart rate during 1 hour running protocol

The average sweat loss rate of 1.35 ± 0.25 L/hr and 1.29 ± 0.15 L/hr for probiotic and placebo groups, respectively, was not different (p=0.40). Fluid intake during the 1-hour treadmill run of 0.11 ± 0.17 L/hr and 0.15 ± 0.14 L/hr for probiotic and placebo groups, respectively, was not different between groups (p=0.69). Changes to body mass of participants between the trials were 1.7% and 1.6% for probiotic and placebo groups, respectively.

Oxygen consumption increased from 3.39 ± 0.27 L/min and 3.47 ± 0.32 L/min at 15 minutes to 3.73 ± 0.49 L/min and 3.75 ± 0.51 L/min at the end of the 45-min steady state stage for probiotic and placebo groups, respectively. On average during the 45-min steady state phase, participants were working at 72.9% VO_2max during probiotic trial and 73.5% VO_2max during the probiotic trial, which was not different (p=0.71).

5.4 Running performance

Figure 6 shows the individual distance completed during the 15-min time trial of both trials. The average distance completed during the time trial was 2.98 ± 0.22 km and 3.03 ± 0.30 km for the probiotic and placebo groups, respectively, which was not significantly different (p=0.63). 5 out of the 7 participants showed improvements to time trial running distance during the probiotic trial compared to placebo. The average total distance completed during the 1 hour run (including 45-min preload and 15-min time trial) was 11.30 ± 0.46 km and 11.35 ± 0.62 km for the probiotic and placebo groups, respectively.

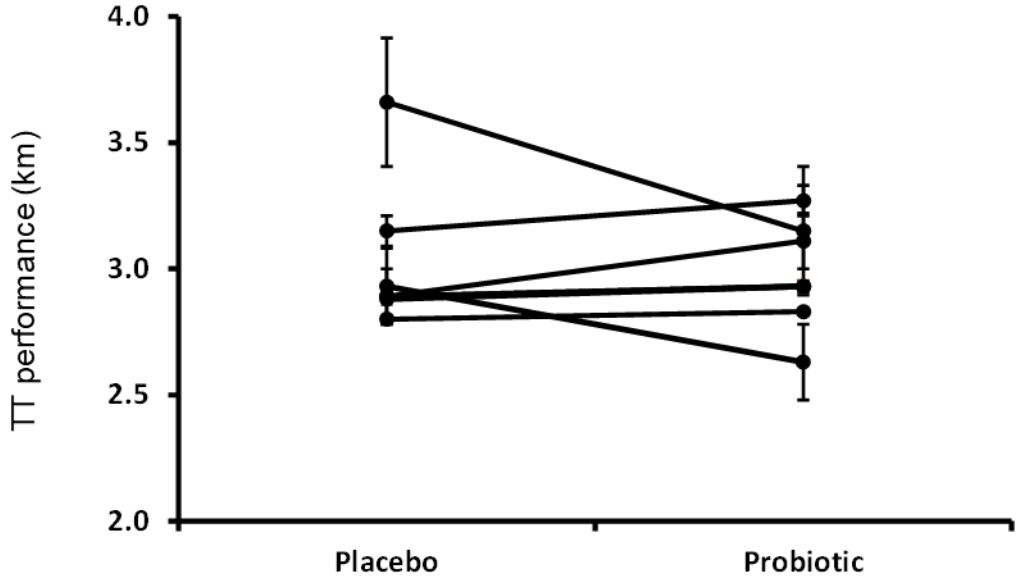

Figure 6. Individual running performance during 15-min time trial

Chapter Six: Discussion

This is the first study to determine the effect of Fonterra's two proprietary probiotic strains, Lactobacillus HN001 and Bifidobacterium HN019, on exercise performance in recreational male runners while under heat stress. The aim of this study was to test the hypothesis that dual probiotic supplementation confers an ergogenic effect on male runners during a preloaded time trial in the heat. Additionally, whether this dual probiotic supplementation would have an effect on physiological measures, including HR, core temperature and fluid loss, was also of interest. The study results rejected both the primary and secondary hypotheses, with no changes found in performance or physiological measures following 4 weeks' probiotic supplementation. Notably, the chosen protocol was robust and produced reliable results as shown by a CV of <5% which is only twice as much as the natural physiological coefficient of variation of 2.4% and 2.5% for HR and VO_2 responses, respectively.

6.1 Study design

The protocol used in the present study was developed based on a previous protocol by Shing and colleagues (Shing et al., 2014). The development process was supported by additional literature to ensure appropriate extrinsic factors, including exercise duration, intensity, mode of exercise, environmental conditions and dehydration, were included as all of these factors are key in their ability to influence the magnitude of disturbances within the gut, thereby increasing the likelihood of inducing gastrointestinal distress through exertional heat stress within participants. This robust protocol needed to maximise the effect of the intervention while also ensuring accuracy of results was achieved.

Exercise induced gastrointestinal syndrome is a common feature of exercise. It is primarily caused by splanchnic hypoperfusion and increased sympathetic stimulation which can induce intestinal epithelial injury, dysregulation of epithelial functioning, malabsorption, hyperpermeability, inflammatory responses, bacterial translocation, and potentially systemic endotoxemia (Costa, Gaskell, McCubbin, & Snipe, 2020). Furthermore, the developed exercise protocol was also required to be achievable for participants to complete under the set conditions.

The present study used warm ambient conditions set at 30°C and 50% RH. Warm ambient temperatures have been shown to add to the effects of exertional stress in participants during exercise. A previous systematic review has suggested core body temperature needs to reach at least 39 °C in order to have an effect on GI status, specifically due to the proportional effect on intestinal hyperpermeability as core temperature rises above this thermoregulatory threshold (Costa et al., 2017). Both trial groups in this present study were shown to have an average core temperature of approximately 39.3°C during the 15-min time trial stage. Therefore, the environmental settings were appropriate for augmenting potential GI disturbances.

Exercise mode can impact on incidence and severity of gastrointestinal syndrome (GIS) (Pfeiffer et al., 2011; van Nieuwenhoven, Brouns, & Brummer, 2004). Running is thought to disrupt the gut due to physical jarring and mechanical stress as a result of the repetitive vertical oscillation (de Oliveira, Burini, & Jeukendrup, 2014). GI stress is often a

characteristic within endurance runners, therefore running was chosen as the mode of exercise most likely to cause additional stress to the gut.

The original protocol by Shing et al. (2014) implemented a mixed trial consisting of 30-min steady state at 80% VT followed by a 5km time trial. During pilot testing for the present study, it was identified that recreationally trained runners were unable to complete the protocol as physical and physiological (core temperature) limits were reached. Therefore, the experimental performance test was adjusted to consist of 45-min steady state and 15-min time trial at 70% VT. This new protocol ensured that the duration of exercise would be 1 hour in the warm conditions. Portal blood flow has been shown to decrease by 80% following 1 hour of cycling at 70% VO_2max (Rehrer, Smets, Reynaert, Goes, & Meirleir, 2001). Running is likely to exacerbate gut symptoms further due to the physical stressors mentioned previously. Therefore, 1 hour running at 70% VT is likely to have an effect on intestinal injury and hyperpermeability due to the increased risk of hypoperfusion and possible gut ischemia. Performance could also be affected as a result of these gut discomforts.

Participants in the present study were shown to be working at approximately 73% VO_2max and 85% HR max during the 45-min steady state period. Costa argues exercise intensity and duration are crucial to exposing participants to sufficient exertional heat stress that intestinal disturbances occur as a result of the body's thermoregulatory system being pushed to its limit (Costa et al., 2020). Running intensity was shown to have a dose dependent effect on intestinal permeability, with 60-min of running (22°C, 50% RH) at 80%VO_2max exacerbating permeability of intestinal epithelium compared to 40% and 60% VO_2max (Pals, Chang, Ryan, & Gisolfi, 1997).

Additionally, participants were observed to experience cardiac drift of approximately 20 bpm, and a 2.5°C increase in core temperature over the duration of 60-min run. The physiological data highlight the impact of exertional heat stress on thermoregulatory mechanisms during exercise. The cardiac and core temperature drift were seen to be steadily increasing at the point of cessation with no sign of plateau which indicates participants were showing signs of physiological stress all the way to the end of the protocol. Furthermore, despite these substantial physiological responses to the experimental trial, participants were able to complete the protocol and remain within the ethical limits for core temperature, a feat unachievable using the 30-min steady state, 5 km time trial protocol at 80% VT during pilot testing.

Hydration status was another study characteristic that was controlled within this study protocol. The risk of dehydration during exercise is heightened with the addition of heat stress owing to increasing sweat rate (Costa et al., 2020). It is believed dehydration can play a part in GIS incidence due to increased internal temperature and subsequent blood flow diversion to the skin, possibly resulting in hypoperfusion of the gut. In the case of severe dehydration, hypoperfusion of the gut can also be affected by a lower plasma volume. Evidence has shown dehydration prior to 90 minutes of cycling at 70% VO_2max can have a significant effect on GIS and delay gastric emptying (van Nieuwenhoven, Vriens, Brummer, & Brouns, 2000). Likewise, following 1 hour of running with no fluid intake, intestinal permeability increased compared to when at rest, while the same study also reported 1.5%

body mass loss in the no fluid trial compared to negligible body mass change to both water only and glucose solution trial groups (Lambert et al., 2008). In the present study, body mass loss was similar at 1.6 -1.7% following 1 hour of running. Therefore, as hydration status can impact gut disturbances there was a need to minimise this confounding factor. Participants were euhydrated, determined by USG readings less than 1.020, prior to the trial. Water was also provided at a rate of 1.5 mL/kg every 15 minutes to ensure dehydration did not occur during the hour run, although only a minimal amount (110 mL and 150 mL for probiotic and placebo groups, respectively) was consumed which reflects their euhydrated state. Thus, standardising hydration within this study protocol mitigates any effect dehydration may have on gut status and subsequent symptoms and influence on performance.

Although dietary intake and physical activity over 2 – 3 days was recorded as part of lifestyle standardisation, it should be acknowledged that self-report tools have been previously linked with a common bias for reporting errors, specifically under-reporting of dietary intake and misreporting of physical activity logs (Prince et al., 2008; Ravelli & Schoeller, 2020). Underreporting of dietary intakes has been linked to errors in measurements. Similarly, it appears that the self-reported records in the present study have followed this same pattern with energy intake lower than expected for active male population compared to their supposed energy expenditure. It has been suggested that a male endurance athlete could expend roughly between 12,500 – 20,000 kJ/day depending on age, training load, and body size and composition (Thomas, Erdman, & Burke, 2016) . Evidently, the macronutrient intakes per kg body mass of the participants in the present study were more similar to general population guidelines rather than athlete specific guidelines.

Notably, the CHO g/kg intakes of the participants were on the lower end of the recommendation for a general active population (3 – 5 g/kg/day), protein intake per kg was slightly higher than general guidelines of 0.81 – 1.2 g/kg/day, while fat intakes were within the expected range (0.5 g/kg/day) (Kerksick et al., 2018). It has been reported previously that protein is the least underreported macronutrient, although it is unknown which foods are more at risk if being under reported (Ravelli & Schoeller, 2020). Overall, more importantly, there was no significant difference between individual daily macronutrient intakes (g/kg), or total daily energy intake (kJ) of probiotic and placebo groups. Arguably it appears that despite the discrepancies from misreporting the dietary or energy expenditure records would not have any effect on the overall study results.

6.2 Interpretation of results

This study found no performance or physiological effects following 4 weeks of probiotic supplementation compared to the placebo group. Overall, the magnitude of the intervention effect was not greater than CV of <5%, as performance during the probiotic trial decreased by 1.2% ± 7.4% compared to placebo trial which highlights the large performance variation among participants. Despite the absence of a significant performance result, 5 out of 7 participants improved their TT performance during the probiotic trial which is a somewhat promising result. While it is acknowledged that the study was underpowered with only seven participants, it is important to note this study is part of a wider, larger project that aims to recruit 27 participants to ensure statistical power is ultimately achieved. Therefore, although no overall significant performance change can be reported in this thesis, this study has identified 5 out of 7 participants improved performance following

HN001 and HN019 supplementation and has thus made a significant start to the wider project in investigating these proprietary probiotics in a novel setting.

It is difficult to compare the results from the current study to those previously published as no other study has investigated *L. rhamnosus* HN001, *B. lactis* HN019, or a combination of both in this exercise performance setting. As mentioned throughout, the efficacy of probiotics is hugely strain and species specific. Thus, findings from one study cannot be crudely extrapolated to strains or species different to that of the original study. Furthermore, different exercise protocols, including exercise mode, duration and intensity, alongside varying probiotic dose, delivery vehicle and wash out period, can also have an impact on the efficacy of intervention, and therefore the outcome of the study.
Of the six studies that have previously reported significant changes to aerobic exercise performance measures following probiotic intervention, five reported exhaustive aerobic exercise time measures(Huang et al., 2018; Huang, Lee, et al., 2019; Huang, Pan, et al., 2020; Huang, Wei, et al., 2019; Shing et al., 2014) whereas the remainder used the timed Cooper test as the performance measure (Salarkia et al., 2013; Salehzadeh, 2015). Although slightly different protocols were used amongst the previous studies compared to the present study, all were aerobic performance-based protocols, therefore are valid for comparison with the present study. In an attempt to normalise the difference in measures between the range of performance tests, percentage change between intervention and placebo trial can be assessed within studies, permitting standardised comparisons to be made.

Previous literature has reported mixed results for probiotic interventions with runners. Shing et al. (2014) investigated male running performance in the heat following probiotic supplementation as part of a cross over study. Four weeks of multi strain probiotic supplementation at 4.5×10^{11} CFU increased run time to fatigue in warm conditions (Shing et al., 2014). This equated to 12.5% improvement in performance outcome. Whereas, a placebo-controlled trial reported 4.5% improvement to Cooper test performance in the yoghurt probiotic group, and a 4.2% decrement to performance in the placebo group (Salehzadeh, 2015). Performance improvement of 31% was reported for a study using an exhaustive Bruce protocol (Huang, Pan, et al., 2020). Exhaustive cycling performance also improved by 25% following 4 weeks PS128 3×10^{10} supplementation (Huang, Wei, et al., 2019). It appears that the two studies that used non-trained participants reported greater percentage changes to performance, with 36.7% improvement to exhaustive run time (Huang et al., 2018), and a significant dose dependent improvement of 17% and 31% for the low and high dose groups, respectively (Huang, Lee, et al., 2019). This could be from probiotic supplementation having greater efficacy in non-trained participants, possibly due to the initial poorer status of gut microbiota composition which has been shown to be improved in active and trained populations. Therefore, it is a possibility that the composition of microbiota of the participants in our current study was balanced due to their active, and trained lifestyles, resulting in less opportunity for improved performance responses via a possible diversification of gut microbiota, hence no significant performance outcomes were observed.

Participants in the current study represented a healthy, active, and recreationally trained population. Previous literature has shown athletes have an improved microbiome

composition (Jang et al., 2019; Monda et al., 2017), with one study reporting a greater diversity of microbiota in athletes compared to healthy controls (Clarke et al., 2014). Subsequently, the participants involved in this study may have presented with optimal microbiome diversity, therefore additional probiotic supplementation would not have had any noticeable effect on microbial interactions within the host. The wider study will be assessing microbiome composition using faecal and throat analysis, however until those results are reported, it could be assumed that the absence of dysbiosis in the participants and therefore lack of altered microbial interaction or subsequent inflammatory response may be the reason that no performance effect was found.

While unable to determine significant improvements directly to performance, several other studies observed promising results. Salarkia et al. (2013) did not observe improvement to 400m swim time, however VO_2max, as determined by Harvard step test, was significantly improved. Interestingly, the present study did not observe any significant changes to VO_2 responses between groups during the steady state phase of the run. Similarly, two other studies also did not report improvements to run time, although improved pace during the last leg of a marathon and improved times at the 6^{th}, 9^{th}, and 12^{th} minute were promising outcomes following probiotic supplementation.

It is difficult to identify the reason for the lack of significant results in the current study, however it can be assured that the study protocol was likely not a contributing factor owing to the robust design mentioned previously. All studies that described beneficial findings used a supplementation period of at least 4 weeks which ensures adequate time for the live bacteria to colonise within the gut. Likewise, the present study also utilized a 4-week supplementation period. It has previously been suggested that intervention studies of less than two weeks should be considered with caution as 14 days of supplementation is minimum time for adequate colonization and adaptation of the host ((Coqueiro et al., 2017; Pyne et al., 2015)

While the supplementation period was at least 4 weeks, probiotic dosage may be a reason for the mixed results reported across studies. The International Society of Sports Nutrition (ISSN) report on probiotics in sport found the typical dose range for *Lactobacillus* and *Bifidobactrium* strains to be 1 x 9^{10} – 10 x 10^{10} CFU and 7 x10^7 – 9.5 x 10^{10} CFU, respectively. Likewise, the studies identified in Chapter 2 to observe improved performance also reported dosage regimens within this range, although a relatively low dose of 2.10 x 10^7 was noted for a study by Salehzadeh (2015) and Huang et al. (2018) implemented a dose protocol of 1 x 10^{11} CFU/day. Likewise, the present study used 1.2 x 10^{10} billion CFU/day, however, this was a dual strain probiotic including 6 x 10^9 CFU/day *Lacticaseibacillus rhamnosus* HN001 and 6 x 10^9 CFU/day *Bifidobacterium anamalis* ssp. *lactis* HN019. In contrast, the only other study to report improvements to performance in the heat used 4.5 x 10^{10} billion CFU of a multistrain which included *Bifidobacterium lactis*, *Lacticaseibacillus rhamnosus* and *Limosilactobacillus fermentum* strains (Shing et al., 2014). This is over 3.5-fold greater dosage of live microorganisms compared to the present study and may account for some of the performance differences reported. In addition, the *Lactobacillus* strain (details not provided) included in Shing's multistrain regimen was over twice the *L. rhamnosus* dose in the current study. Furthermore, several animal model studies have outlined significant dose dependent effects on performance and physiological outcomes (Chen et al., 2016; Hsu et al.,

2018; Lee et al., 2019; Lee et al., 2020). The one human study to use a dose dependent protocol also found significant dose dependent improvements to endurance performance (Huang, Lee, et al., 2019). Therefore, there is reason to believe a dose dependent effect is possible for certain probiotic strains and more research is required to elucidate the optimal dose regimens for performance enhancing benefits.

Evidence behind the delivery methods of the probiotic intervention is limited. Most previously published protocols have implemented delivery via freeze dried probiotic powders in the form of daily capsules. However, several protocols have also used a range of other delivery vehicles including kefir, yoghurt, powder mixed with water, milk-based drink, and beverages. While previous protocols have achieved success in using daily probiotic capsules, probiotic yoghurt as a delivery vehicle has also been involved in two studies that reported beneficial changes to aerobic exercise performance and VO_2max (Salarkia et al., 2013; Salehzadeh, 2015). This suggests there is no effect of probiotic delivery on efficacy known at this time. Thus, no clear pattern has emerged in regard to probiotic delivery vehicle and the decision for more studies to use capsule vehicles at this stage may be owing to the more practical components of administering, storing and analysing capsules.

Running was chosen as the preferred mode of exercise for the protocol due to the additional strain on the gut that is experienced from the physical jarring and vertical oscillatory motion of running. Interestingly, of the 16 studies that have previously investigated aerobic performance outcome and probiotic intervention, 11 studies used running based protocols to assess performance outcomes. Five of these studies make up the 6 studies that did report improvements to performance, with the other study using cycle ergometer protocol. Furthermore, while two more running based studies were unable to report direct significant changes to performance, they observed promising changes including improved run times at 6^{th}, 9^{th}, 12^{th} minute, as well as improved pace during the last leg of a marathon. The rest of the 12 studies unable to report any significant performance outcomes were made up of cycling (5), swimming (3) and running (4). This breakdown indicates that mode of exercise can play a critical role in gut disturbances, and running is likely the most appropriate protocol to induce GIT disturbances and therefore optimise the likelihood of the intervention conferring any effect.

Despite the evidence which highlights the effect of heat stress, additional to exercise stress, on gut functioning, only two previous studies have implemented a heat stress protocol combined with exercise (S. K. Gill et al., 2016; Shing et al., 2014). Heat stress, with or without exercise, has been shown to exacerbate GIS (Costa et al., 2020). Several proposed mechanisms for how probiotics confer beneficial performance effects on host health are strongly linked with the gut, which underlines how additional heat stress can further augment these detrimental changes to gut epithelial integrity, microbiota composition, and subsequent disruption to gut inflammatory and immune function. The study by Shing et al. (2014), conducted at 35°C, 40% RH, reported improvement to exhaustive run time, however no changes to physiological measures including HR or core temperature were observed between groups. No difference between probiotic and placebo groups for HR or rectal temperature were reported following 2 hours running at 60% VO_2max (34°C, 32% RH) (performance details not reported) (S. K. Gill et al., 2016). Likewise, the current study also did not find any difference between HR or core temperature following 1 hour exercise at

70% VT (30°C, 50% RH). It can be assumed that the lack of heat stress protocols may be due to the practical and cost implications of using heat chambers. Although the three heat stress studies have mixed results, this could be because of the mixed exercise protocols used, and as mentioned above, the experimental trial factors, such as exercise duration, and probiotic intervention protocol can play a critical role in the success of results. Likely, a 7-day supplementation period, together with a submaximal (60% VO_2max) 2 hour running exercise protocol was not strenuous enough to cause a rise in rectal temperature to be greater than 39°C, thus was not sufficient to induce physiological changes in athletes (S. K. Gill et al., 2016). Furthermore, it could be implied an approximately 37-minute running duration at 80% VT was also insufficient to adequately induce GIS (Shing et al., 2014). This is because evidence suggests an exercise duration of more than 2 hours is essential for GIS inducing protocols as core temperature needs to be >39°C in order to exceed human thermoregulatory responses and have an effect on GI status (Costa et al., 2017). While core temp may have reached > 39°C during the time trial of current study and during the exhaustive run of Shing's study, longer duration exercise protocol may have had more of an impact on GI status, increasing the likelihood of physiological changes as a consequence of the heat and exertional stressors.

As mentioned earlier, participants were euhydrated and despite being offered 1.5ml/kg/15 min of water during the run, they consumed very little (approx. 100 – 150 mL). The water intake consumed during the trial, as well as the fluid loss (approximately 1.6%) were not different between groups. This fluid protocol was like that of a previous protocol (Shing et al., 2014) who also reported similar body mass loss of approximately 1.5%. Interestingly, the other heat stress study observed similar body mass loss of 1.3 – 1.5%, however these participants ingested ad libitum amounts of 1.7 - 1.9 L during the 2-hour running session. This pattern supports comments from participants who mentioned anecdotally they would not consume water during run sessions < 1 hour in duration since runners often refrain from drinking due to the practical implications and the nature of vertical motion. This meant, although participants were well hydrated at the start of the trial, by the end, mild dehydration had set in with an average of 1.7% and 1.6% loss in body mass for probiotic and placebo groups, respectively. Importantly, this fluid loss was not different between groups, thus the proposed impact on the results of the study are unlikely.

6.3 Strengths and limitations

This study had several strengths and limitations. The strength of this study is supported by the soundness of the design. The design consisted of double blinding and placebo-controlled study to reduce risk of bias during data collection. Researchers and participants were blinded to the randomised order allocation for probiotic and control groups. Blinding both parties until completion of the study period ensured there was no selection bias or placebo effect.

The washout duration was 3 weeks which is adequate time for the gut microbiota to return to the pre-supplementation levels and ensured no effects of intervention would be carried over. Previous work has shown levels of *Lacticaseibacillus rhamnosus* HN001 in faecal samples following 4 weeks of cheese supplementation (1×10^9 CFU/day) return to pre-intervention levels following 4 weeks wash out period (Sheih et al., 2001). Ahmed and colleagues showed 2 weeks washout following 4 weeks of HN019 at varying dosages was

insufficient in elderly populations (Ahmed et al., 2007), however, another study reported 4 weeks wash out allowed faecal counts of *Lactobacillus* and *Bifidobacterium* counts to return to pre intervention levels in healthy participants aged 20 - 60 years after 2 weeks HN019 supplementation at 3 x 10^{10} CFU/day (Gopal & Gill, 2003). Additionally, following a 3-week washout levels of immune markers returned to pre intervention levels after 3 weeks of both low (5 x 10^{9} CFU/day) and high (5 x10^{10} CFU/day) dose HN019 supplementation (Harsharnjit S Gill, Rutherfurd, Cross, & Gopal, 2001). Therefore, 3 weeks wash out was determined to be sufficient for the dual probiotic protocol implemented in the current study.

Another strength of this study is the use of time trial protocol compared to a time to exhaustion performance protocol. Literature has reported lesser variability for performance testing outcomes from time trial protocols as opposed to time to exhaustion tests (Laursen et al., 2007). Time trials provide a testing opportunity that is more likely to represent a real-life racing event therefore the validity of the results is improved. The variation of results is also shown to be reduced compared to reliability results from open-ended time to exhaustion tests. Additionally, the inclusion of a familiarisation trial allowed any learning effects to be minimised during the following test and retest trials. As outlined in Table 7, CV was shown to be 5.29% between the familiarisation trial and trial 1, with TT variation reduced to 4.26% between the last two trials.

Recruitment and testing for this 12-week study were conducted in the late winter through to early summer months, thus participants were non-acclimated during each trial. There is evidence to suggest heat acclimation can have a significant influence on physiological adaptations which can affect performance outcomes in both temperature and warm conditions (Lorenzo, Halliwill, Sawka, & Minson, 2010). For example, cardiovascular responses, such as stroke volume and cardiac output, were shown to improve following a 10-day heat acclimation period, with positive changes to aerobic performance observed as a direct result. By restricting the recruitment period in the present study, the risk for acclimation effects has been mitigated.

While this was a sound study, there were limitations. This study and results are limited by the number of participants involved. Due to COVID-19 restrictions, the period of recruitment was considerably shortened, leading to a smaller number of participants completing the study. This issue was further exacerbated by the considerably long study period and the participant burden to meet study conditions, such as avoid certain probiotic dietary items and antibiotics, for the duration of the 12 weeks.

A possible disadvantage of the study design may have been the 45-min fixed intensity preload followed by the time trial. There may be concern that this combination of performance protocols could cause participants to reach their physiological limits, especially under heat stress. However, adopting a combination of steady-state preload and TT allows for steady state data to be collected whilst obtaining maximal performance data within the same trial. During pilot testing, it was observed that the original protocol consisting of a 30-min preload combined with a 30-min time trial was too great an intensity for participants to complete without reaching physical exhaustion and/or heat stress prior to the end of the time trial. Thus, considerations were made to adapt the protocol to consist of 45-min fixed intensity preload followed by a shortened 15-min time trial. Based on the physiological data

for the preload stage, participants were working at approximately 75% VO_2max and 83-85%HRmax which suggests while physiologically participants were working hard, they were not at their physiological limit leading into the time trial, therefore were capable of completion.

6.4 Considerations

Whilst supplementation could continue throughout alert level changes, COVID restrictions impacted testing abilities. This meant participants on supplementation were required to continue supplementing, thereby extending days on supplementation past the original 4-week protocol. Difficulties arose when these prolonged supplementation periods (of up to 60 days) were matched in length during Phase 2 supplementation. Therefore, an 11-week study period was closer to a 5-month study for several participants, which significantly increased participant burden and influenced research timelines. Participant recruitment was also affected by COVID restrictions as recruitment was severely delayed. As a result, the original target of 12 participants was not achieved. Accordingly, it has proved crucial that reliability data was obtained to highlight the soundness of the study despite the adversities that were faced by the research team and participants during this study in 2020.

This study is limited by its male only subjects. Recruitment was limited to males as the female gender has shown to have significant hormonal differences to male population that can affect exercise physiology. Female hormonal status can influence core temperature, with increases of 0.3°C during luteal phase (Baker, Siboza, & Fuller, 2020). As this study was conducted within warm ambient conditions and looking directly at core temperature as a physiological measure, female runners would have added further complexity to this already detailed, novel wider study. In the future, if these hormonal differences are controlled, specifically tracking the menstrual cycle, and determining hormone levels, female runners may be an appropriate subject group. Furthermore, there is also evidence to suggest greater incidence and severity of GI symptoms in females relating to premenstrual and menses phases of the menstrual cycle, thus another reason to need to track the menstrual cycle as the wider study will be investigating GI symptoms and severity during the 1 hour run (Bernstein et al., 2014; Heitkemper & Jarrett, 1992). Additionally, it should be noted that unrelated to menstrual cycle, women experience more GI symptoms (van Nieuwenhoven et al., 2004). This is apparent in females with inflammatory conditions, such as endometriosis, where evidence suggests females experience more severe GI symptoms, including cramping, constipation, abdominal pain and intestinal symptoms (Ek et al., 2015). Therefore, the results of this study are relevant only to male recreational runners and caution should be taken when generalising to female populations.

6.5 Recommendations and practical applications

In light of our findings, there are several areas to consider for future research. A larger sample size would ensure the study is statistically powered. This ensures the margin of error is reduced, and the effect of intervention may be more clearly observed. There should be consideration of including a dose dependent protocol for future studies in this area, specifically with a higher dose than used in the current study (1.2×10^{10} CFU/day). Several animal model studies have reported dose dependent improvements to aerobic performance tests such as swim and run time (Chen et al., 2016; Hsu et al., 2018; Lee et al., 2019; Lee et al., 2020). The only dose dependent study in humans published in this space reported an increasing trend for performance benefit with the highest dose of 9×10^{10} CFU/day (Huang,

Lee, et al., 2019). Therefore, there is room to increase the dosage and implement a dose dependent study design in the future to identify any dose dependent effects of these two proprietary strains.

It may also be beneficial to consider conducting separate trial groups for each probiotic. There is evidence to suggest synbiotic relationships between certain prebiotic and probiotics (Markowiak & Śliżewska, 2017), however this could also apply to possible synergistic interactions between different probiotics (Chapman, Gibson, & Rowland, 2011). A review concluded that although difficult to compare in some cases, it is possible that multistrain probiotic interventions may have greater efficacy over single strain. Notably, authors also suggested that the efficacy of *Bifidobacterium* strains maybe inhibited by the presence of other bacteria within a multistrain intervention. Hence, separate, or combined effects of HN001 and HN019 would be able to be determined by separating the two probiotics into two study groups.

Interestingly, a study by West et al. (2011), reported inconsistencies between female and male immune responses following probiotic supplementation. Additionally, changes to gut microbiota in males following probiotic supplementation were notably absent in the female group. Further investigation is required to pursue this area of probiotic intervention studies. If females were included within recruitment criteria, additional steps would need to be taken to standardise the effect of hormones on exercise performance and core temperature during different phases of menstruation. Results from these future studies may well have clinical significance for future advice on probiotic supplementation for athletes.

Chapter Seven: Conclusions

Following 4 weeks' supplementation, there was no evidence to suggest *L. rhamnosus* HN001 HN001 and *B. lactis* HN019 are effective as a nutritional ergogenic aid for male recreational runners under heat stress in exercise of 1 hour. The impact of these probiotics on physiological adaptations during the experimental trial was also negligible.

As previously mentioned, the current study developed a sound protocol that aimed to combine relevant lab conditions in order to optimise the likelihood of gut perturbations, meaning the efficacy of the intervention was maximised. The protocol considered appropriate intervention parameters with adequate supplementation (duration and dose), alongside relevant exercise mode, duration, intensity, and environmental conditions. In comparison to other studies that have reported both beneficial performance outcomes and physiological outcomes, the current study has not been hindered by the absence of a robust study design. Instead, it is likely the lack of significant performance and/or physiological outcomes are due to three critical reasons: (1) the study was underpowered, (2) the dose was insufficient for these two strains in the current setting, or (3) combined supplementation of HN001 and HN019 strains do not act as an ergogenic aid or effect physiological outcomes. However, the first two points need to be explored further before confirming the third possibility.

Overall, more research is required in this area to further determine the efficacy of *L. rhamnosus* HN001 and *B. lactis* HN019 as performance enhancing supplements. Dependent

on funding and resources, a future study should consider several improvements to the design, including:

- Dose dependent relationship
- Increased sample size
- Interaction between multi strain probiotic supplements
- Impact of sex on probiotic induced outcomes

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